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(54) Title: RECOMBINANT VACCINES COMPRISING IMMUNOGENIC ATTENUATED BACTERIA HAVING RPOS POSITIVE PHENOTYPE

(57) Abstract

Attenuated immunogenic bacteria having an RpoS+ phenotype, in particular, *Salmonella enterica* serotype Typhi having an RpoS+ phenotype and methods therefor are disclosed. The *Salmonella* have in addition to an RpoS+ phenotype an inactivating mutation in one or more genes which render the microbe attenuated, and a recombinant gene capable of expressing a desired protein. The *Salmonella* are attenuated and have high immunogenicity so that they can be used in vaccines and as delivery vehicles for genes and gene products. Also disclosed are methods for preparing the vaccine delivery vehicles.

RECOMBINANT VACCINES COMPRISING IMMUNOGENIC
ATTENUATED BACTERIA HAVING RPOS POSITIVE PHENOTYPE

Background of the Invention

(1) Field of the Invention

This invention relates generally to attenuated

5   microbes and, more particularly, to novel attenuated
bacteria having an RpoS[+] phenotype for use as vaccines and
delivery vehicles for genes and gene products and to
methods for their preparation. This invention is
particularly applicable to Salmonella such as Salmonella

10 enterica serotype Typhi (also referred to as Salmonella
typhi).

(2) Description of the Related Art

Live attenuated Salmonella strains can serve as
delivery vehicles for recombinant antigens or other

15 proteins. As antigen carriers, the recombinant
Salmonella have been shown to be useful in live vaccines
(For review see Curtiss et al. in Essentials of Musocal
Immunology, Kagnoff and Kiyono, Eds., Academic Press, San
Diego, 1996, pp. 599-611; Doggett and Brown, in Mucosal

20 Vaccines, Kiyono et al., Eds., Academic Press, San Diego,
1996 pp 105-118; see also Hopkins et al. Infect Immun.
63:3279-3286, 1995; Srinavasin et al Vaccines 95,

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R.N.Chanock et al., Eds., Cold Spring Harbor Laboratory
Press, Plainview, NY, p 273-280, 1995).

Ideally, live attenuated vaccine strains should
possess a balance between the two properties of
5   attenuation and immunogenicity. Such vaccine strains
would not cause any disease or impair normal host
physiology or growth, thus being attenuated, and at the
same time be able to colonize the intestine and gut
associated lymphoid tissue upon oral administration or
10   other lymphoid organs upon administration by some other
route so as to be immunogenic. As a practical matter,
however, such an ideal balance has not been achieved
(Curtiss, in *New Generation Vaccines* Woodrow and Levine,
Eds., Marcel Dekker, Inc., New York, 1990, pp. 161-188).
15   This may be a result of the almost exclusive focusing of
efforts in *Salmonella* vaccine development on improving
the attenuation component of strains rather than on
producing strains with high immunogenicity.

Work directed toward achieving attenuation in
20   microbes for use in vaccines has utilized attenuating
mutations in biosynthetic genes, regulatory genes and/or
genes involved in virulence. (See Doggett and Brown,
supra). One such regulatory gene which has been mutated
as a means for achieving attenuation has been the *rpoS*
25   gene. The *rpoS* gene encodes an alternative sigma factor,
RpoS, which is known to regulate the stationary phase
expression of over 30 genes (for review, see Loewen and
Hengge-Aronis, *Annu Rev Microbiol 48*:53-80, 1994). The
rpoS gene has been shown to contribute to the virulence
30   of *Salmonella enterica* serotype Typhimurium (also
referred to as *Salmonella typhimurium*) in mice by RpoS
regulation of chromosomal as well as plasmid-borne genes

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(Fang et al., *Proc Natl Acad Sci 89*:11978-11982, 1992;
Norel et al., *FEBS Microbiol Lett 99*:271-276, 1992;
Kowarz et al., *J Bacteriol 176*:6852-6860, 1994).
Similarly, RpoS is thought to contribute to the virulence

5   of *Salmonella typhi* in humans by an action on chromosomal
gene determinants of virulence, inasmuch as these
microbes do not possess the virulence plasmid present in
S. typhimurium (Robbe-Saule et al., FEMS Microbiol Let
126:171-176, 1995; Coynault et al., *Mol Microbiol 22*:149-

10 160, 1996). Mutant *rpoS S. typhimurium* strains have been
shown to be attenuated (Fang et al, *supra*) and capable of
eliciting protective immunity in mice (Nickerson and
Curtiss, *Abstracts of the 96th General Meeting of the
American Society for Microbiology B-141*:179, 1996;

15 Coynault et al., *Mol Microbiol 22*:149-160, 1996). As a
result, it has been suggested that *rpoS* mutants may be
attractive candidates for the development of vaccines
(Nickerson and Curtiss, *supra*).

Attenuated strains of *Salmonella typhi* have been

20 used as human vaccines against typhoid fever as well as
against heterologous antigens when used as recombinant
antigen delivery vehicles (Forrest, in CRC Press Inc.,
1994, pp. 59-80; Levine et al, in *New Generation Vaccines*
Woodrow and Levine, Eds., Marcel Dekker, Inc., New York,

25 1990, pp. 269-287). These vaccines based upon Typhi
strains have almost exclusively been derived from the Ty2
strain, in particular, Ty21a, which contains a *galE*
mutation along with other mutations. Ty2 and its Ty21a
derivative vaccine strain have been shown to be *rpoS*

30 mutants and this mutation may account, at least in part,
for the attenuation seen with Ty21a and with other
vaccine strains derived from Ty2 presumably by the down

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regulation of chromosomal virulence genes controlled by
the *rpoS* gene product. The Ty21a vaccine is typical of
vaccines derived from Ty2 in that although being
attenuated, the Ty21a vaccine has proven to have low
5   vaccine efficacy, requiring three high doses of
approximately 10^{10} cfu to induce protective immunity in
approximately two-thirds of the vaccinated individuals.
(Forrest, *supra*). Thus, there remains a continuing need
for *Salmonella typhi* strains which exhibit not only low
10 virulence, but, also high immunogenicity for use in
vaccines suitable for the delivery of a desired gene
product to a host.

       Other strains of *S. typhi* have been reported which
may, however, have a functional *rpoS* gene although this
15 was not appreciated at the time of the report. For
example, human vaccines have been reported based upon the
27V and ISP1820 strains (Reitman, *J Infect Dis 117*:101-
107, 1967; Levine et al., *J Infect Dis 133*:424-429, 1976;
Tacket et al., *Infect Immun 60*:536-541, 1992). Neither
20 of these strains contained a recombinant gene nor were
they used to deliver a recombinant gene in a vaccine
composition.

       In a report of recombinant *rpoS*[+] *S. typhi*,
Coynault et al. disclosed the construction of a Ty2
25 derivative containing a recombinant *rpoS* gene which gave
the microbe an RpoS[+] phenotype. However, this Ty2
derivative was used only in a laboratory study and no
additional recombinant gene was incorporated nor was
there any teaching of the use of this derivative in a
30 vaccine composition.

       Finally, the *S. typhi* strains ISP1820 and ISP1822
(U.S. Patents 5,387,744 and 5,294,441 and PCT application

WO/9424291) and the *S. typhi* strain 531Ty (U.S. Patent
4,837,151) have been used to construct derivative vaccine
strains. Although the studies reported herein show
ISP1820, ISP1822 and 531Ty to be RpoS⁺, this was not known
at the time of these earlier publications. Furthermore,
none of these references recognized the importance of the
presence of a functional *rpoS* gene in achieving a high
immunogenicity in a vaccine preparation. As a result,
these references did not disclose the selection of
vaccine strains based upon the presence of an RpoS⁺
phenotype.

All references cited in this specification either
supra or *infra* are hereby incorporated by reference. The
discussion of the references herein is intended to
summarize the assertions made by their authors and no
admission is made as to the accuracy or pertinency of the
cited references or that any reference is material to
patentability.

Summary of the Invention:

In accordance with the present invention, the
inventors herein have succeeded in discovering the
critical importance of a functional *rpoS* gene in
Salmonella vaccine strains in that the presence of a
functional *rpoS* gene and an RpoS⁺ phenotype confers upon
the *Salmonella* the property of high immunogenicity. As a
result, when the RpoS⁺ phenotype is present with one or
more inactivating mutations other than a mutation in an
rpoS gene, which render the microbe attenuated, a new and
advantageous balance of attenuation and high
immunogenicity is achieved. This invention is
particularly applicable to *S. typhi* based vaccines,

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however, it is also applicable to other *Salmonella* such as *S. paratyphi A, B* and *C* as well as to other serotypes of *S. enterica* such as Typhimurium, Enteritidis, Dublin and Choleraesuis. The invention is also applicable to
5 other bacteria having an *rpoS* gene, or functional equivalent thereof, that can colonize human tissues, including *Shigella*, *E. coli*, and hybrids between such bacteria, such as *Salmonella-Shigella* hybrids, *Salmonella-E. coli* hybrids or *Shigell-E. coli* hybrids.
10   In one embodiment of the present invention, a method is provided for delivery of a desired gene product to a human. The method comprises selecting a strain of bacteria such as *S. typhi* on the basis of the strain having (i) an RpoS$^+$ phenotype, (ii) one or more
15 inactivating mutations which render the strain attenuated, and (iii) a recombinant gene encoding the gene product. The selecting step with respect to RpoS$^+$ phenotype can involve, in whole or in part, testing the strain to determine its RpoS phenotype. The strain thus
20 selected is then administered to the human. The one or more inactivating mutations which render the strain attenuated can involve a mutation in one gene or a mutation in each of two or more genes.
        The RpoS$^+$ phenotypic activities of the *Salmonella*
25 or other bacteria can be produced by a chromosomal *rpoS* gene and/or by a recombinant gene introduced into the strain. Thus, in another embodiment, the method comprises administering to a human a live attenuated strain of bacteria having (a) an RpoS$^+$ phenotype, (b) a
30 recombinant *rpoS$^+$* gene, (c) one or more inactivating mutations which render said microbe attenuated and (d) a second recombinant gene encoding the desired product. By

recombinant *rpoS⁺* gene or wild-type *rpoS* gene it is meant that the *rpoS* gene is capable of producing a functional *rpoS* gene product.

The attenuated microbes of the present invention contain at least one recombinant gene capable of expressing a desired gene product, which allows their use as carriers or delivery vehicles of the gene product to humans. Examples of gene products deliverable by the microbes of the invention include but are not limited to: antigens, which can be from a human pathogen, or, for use in autoimmune applications, from the human itself, such as, for example, a gamete-specific antigen; enzymes that can synthesize antigens such as polysaccharides, lipoproteins, glycoproteins, and glycolipids; allergens of the human; immunoregulatory molecules; and pharmacologically active polypeptides. By delivery of the desired gene product it is meant that either the gene product or the polynucleotide encoding the product is delivered to the human. In embodiments in which the attenuated bacteria contains a recombinant *rpoS* gene, the desired gene product is encoded by a second recombinant gene.

In another embodiment, the present invention provides a method for producing a strain of carrier microbes for delivery of a desired gene product to a human. The method comprises (1) selecting for a strain of *S. typhi* or other bacteria having an RpoS⁺ phenotype; (2) producing one or more inactivating mutations in the RpoS⁺ strain to render the strain attenuated; and (3) introducing into the strain a recombinant gene encoding a desired gene product. The selecting step can involve, in

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whole or in part, testing the strain to determine its RpoS phenotype. Steps 1-3 can be performed in any order.

In a further embodiment, the present invention involves another method for producing carrier microbes for delivery of a desired gene product to a human. The method comprises generating a live attenuated strain of *S. typhi* or other bacteria having (a) an RpoS$^+$ phenotype, (b) a recombinant *rpoS$^+$* gene, (c) one or more inactivating mutations which render said microbe attenuated and (d) a second recombinant gene encoding the desired product.

Another embodiment of the present invention provides a carrier microbe for the delivery of a desired gene product to a human. The microbe comprises a live attenuated strain of *S. typhi* or other bacteria having (a) an RpoS$^+$ phenotype, (b) a recombinant *rpoS$^+$* gene, (c) one or more inactivating mutations which render said microbe attenuated and (d) a second recombinant gene encoding the desired product.

In another embodiment a vaccine is provided for immunization of a human. The vaccine comprises a live attenuated strain of *S. typhi* or other bacteria having (a) an RpoS$^+$ phenotype, (b) a recombinant *rpoS$^+$* gene, (c) one or more inactivating mutations which render said microbe attenuated and (d) a second recombinant gene encoding the desired product.

The present invention also provides in another embodiment, a genetically engineered cell. The cell comprises a live attenuated strain of *S. typhi* or other bacteria having (a) an RpoS$^+$ phenotype, (b) a recombinant *rpoS$^+$* gene, (c) one or more inactivating mutations which render said microbe attenuated and (d) a second recombinant gene encoding the desired product. A method

is also provided for the preparation of a vaccine
comprising mixing the genetically engineered cells with a
pharmaceutically acceptable formulation suitable for
administration to a human.

5   The present invention also provides a genetically
engineered bacteria, in particular S. typhi, containing a
recombinant virulence gene that is regulated by RpoS, or
its functional equivalent, in wild-type bacteria and a
method for using the genetically engineered bacteria for

10   the delivery of a desired gene product to a human. The
recombinant virulence gene is capable of expressing a
gene product that facilitates invasion and colonization
of any of the gut associated lymphoid tissues (GALT),
nasal associated lymphoid tissue (NALT) or the bronchial

15   associated lymphoid tissue (BALT) and the like. The
genetically engineered S. typhi or other bacteria can be
further characterized as having one or more inactivating
mutations which render the microbe attenuated as well as
a second recombinant gene encoding the desired product.

20   In still another embodiment, the present invention
provides a method for assessing the RpoS phenotype as an
indication of the immunogenicity of a bacteria strain,
and in particular, of a Salmonella. It is believed that
many bacterial strains propagated and maintained under

25   laboratory conditions accumulate rpoS mutations. Thus,
it would be useful to provide a method for assessing the
RpoS phenotype of a Salmonella or other bacteria,
particularly for a strain being developed for use in a
vaccine. The method comprises determining the RpoS

30   phenotype of the bacteria by assessing characteristics of
the microbe regulated by RpoS. An increased
immunogenicity is indicated by the presence of an $RpoS^+$

phenotype compared to the immunogenicity of an isogenic strain having an RpoS⁻ phenotype. The isogenic RpoS⁻ strain does not exhibit an RpoS⁺ phenotype, but otherwise has the same genetic background as the test strain.

5   As noted above, the delivery of a polynucleotide encoding the desired gene product to a human is within the scope of the methods and compositions of the present invention. Moreover, each of the embodiments above involving methods and compositions based upon microbes

10   having an RpoS⁺ phenotype are further contemplated to include methods and compositions for the delivery of a gene or portion thereof to the cells of a human. The gene or portion thereof can comprise a eukaryotic expression cassette that contains the genetic

15   information, either DNA or RNA, that is intended to be delivered to cells of the human.

         Thus, in one embodiment of the present invention provides methods for delivery of a gene or portion thereof to the cells of a human. One such method

20   comprises selecting a strain of bacteria such as *S. typhi* on the basis of the strain having (i) an RpoS⁺ phenotype, (ii) one or more inactivating mutations which render the strain attenuated, and (iii) the gene or portion thereof. The gene or portion thereof can be within a eukaryotic

25   expression cassette. The selecting step with respect to RpoS⁺ phenotype can involve, in whole or in part, testing the strain to determine its RpoS phenotype. The method can also comprise delivering to cells of a human, a live attenuated strain of bacteria having (a) an RpoS⁺

30   phenotype, (b) a recombinant *rpoS*⁺ gene, (c) one or more inactivating mutations which render said microbe attenuated and (d) the gene or portion thereof. The gene

or portion thereof can be within a eukaryotic expression
cassette.

The present invention also provides methods for
producing a strain of carrier microbes for delivery of a
5   desired gene or portion thereof to a cell of a human.
One such method can comprise (1) selecting for a strain
of *S. typhi* or other bacteria having an RpoS⁺ phenotype;
(2) producing one or more inactivating mutations in the
RpoS⁺ strain to render the strain attenuated; and (3)
10   introducing into the strain the gene or portion thereof.
The gene or portion thereof can be within a eukaryotic
expression cassette. The selecting step can involve, in
whole or in part, testing the strain to determine its
RpoS phenotype and the steps can be performed in any
15   order. The method can also comprise generating a live
attenuated strain of *S. typhi* or other bacteria having
(a) an RpoS⁺ phenotype, (b) a recombinant *rpoS*⁺ gene, (c)
one or more inactivating mutations which render said
microbe attenuated and (d) the desired gene or portion
20   thereof. The gene or portion thereof can be within a
eukaryotic expression cassette.

The bacteria that can be used for delivery of a
gene or portion thereof can be an attenuated *Salmonella*,
E. coli or *Shigella* or *Salmonella-Shigella* hybrid,
25   *Salmonella-E. coli* hybrid or *Shigella-E. coli* hybrid so
long as the attenuated bacteria lyses to release the
nucleic acid within the target host cell.

Among the several advantages achieved by the
present invention, therefore, may be noted the provision
30   of a carrier microbe which is capable of colonizing and
delivering a desired gene product or a desired
polynucleotide to the gut associated lymphoid tissue if

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administered orally, to the nasal associated lymphoid
tissue if administered intranasally and to other lymphoid
organs if administered by other routes; the provision of
an efficient and inexpensive method for delivery of a
5   nucleic acid molecule to human cells based upon the use
of RpoS$^+$ carrier bacteria cells that lyse and release the
nucleic acid molecule; the provision of vaccine
preparations which are highly immunogenic along with
being attenuated; the provision of methods of delivering
10   a desired gene product or polynucleotide to an individual
by administering the carrier microbe so as to elicit an
immune response; the provision of methods of preparing
RpoS$^+$ carrier microbes and vaccines wherein the vaccines
are not only attenuated but also have high
15   immunogenicity; and the provision of methods for
assessing the immunogenicity of a *Salmonella* or other
bacteria by determining its RpoS phenotype.

Brief Description of the Drawings
20   Figure 1 illustrates the time course of survival
within J774 murine macrophage-like cells of an *rpoS$^+$*
Salmonella typhimurium, χ3339, and an isogenic *rpoS*
mutant *Salmonella typhimurium*, χ4973.
         Figure 2 illustrates the time course of survival
25   within rat bone marrow derived macrophages of an *rpoS$^+$*
Salmonella typhimurium, χ3339, and an isogenic *rpoS*
mutant *Salmonella typhimurium*, χ4973.
         Figure 3 illustrates light micrographs at
approximately 200X magnification (indicated by a 50μm
30   bar) showing normal murine Peyer's patch tissue in Fig.
3A; murine Peyer's patch tissue at one day (Fig. 3B),
three days (Fig. 3C), and five days (Fig. 3D) after

peroral infection with χ4973; and murine Peyer's patch

tissue after peroral infection with χ3339 at one day

(Figs. 3E and 3F), three days (Fig. 3G), and five days

(Fig. 3H) post infection.

5   Figure 4 illustrates transmission electron

micrographs at approximately 2000X magnification

(indicated by a 5μm bar) showing normal murine Peyer's

patch lymphoid tissue (Fig. 4A), and murine Peyer's patch

lymphoid tissue at five days after peroral infection with

10 χ4973 (Fig. 4B) or χ3339 (Fig. 4C).

Figure 5 illustrates transmission electron

micrographs at approximately 2000X magnification

(indicated by a 50μm bar) showing normal murine Peyer's

patch tissue (Fig. 5A), and normal murine Peyer's patch

15 tissue five days after peroral infection with χ4973 (Fig.

5B) or χ3339 (Fig. 5C).

Figure 6 illustrates the construction of plasmid

vectors and bacterial strains with the defined ΔphoPQ23

mutation.

20   Figure 7 illustrates the construction of plasmid

vectors and bacterial strains with the defined ΔasdA16

mutation.

Figure 8 illustrates the expression of the

recombinant HBV core-pre-S protein by

25 S. typhimurium Δcya Δcrp Δasd RpoS⁺ and RpoS⁻ strains

containing Asd⁺ vector, pYA3167, expressing the HBV core-

pre-S antigen constructed by introducing the Asd⁺ vector

into S. typhimurium χ8296 (Δcys Δcrp Δasd) and χ8309

(Δcys Δcrp Δasd rpoS) examined by (A)Coomassie blue

30 stained 12% sodium dodecyl sulfate (SDS) polyacrylamide

gel electrophoresis (PAGE) and (B) Western blot with a

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monoclonal antibody directed to the preS2 epitope with
lanes in both gels as follows: lane 1, molecular markers;
lane 2, χ8296 (Δcya-27 Δcrp-28 ΔasdA16 cfs RpoS⁺); lanes
3 & 4, χ8296 containing pYA3167 (Asd⁺ vector expressing
5   HBV core-pre-S Ag); lane 5, χ8309 (Δcya-27 Δcrp-28
ΔasdA16 cfs rpoS); lanes 6 and 7, χ8309 plus pYA3167 (Asd⁺
vector expressing HBV core-pre-S Ag).

Figure 9 illustrates the induction of antibody
titers to HBV core-pre-S protein expressed by S.
10   typhimurium, SL1344 strains in which mice were orally
immunized with 10⁹ CFU or χ8296 (Δcya Δcrp Δasd RpoS⁺)
containing pYA3167 (Asd⁺ vector specifying HBV core-pre-S)
or the corresponding RpoS⁻ derivative, χ8309 (Δcya Δcrp
Δasd RpoS⁺ containing pYA3167) showing (A) serum IgG
15   antibody titer and (B) IgA antibody in vaginal washings
determined at 4 and 6 weeks after immunization by ELISA
using a recombinant polypeptide representing the full
length pre-S sequence as a coating antigen (n=4).

Figure 10 illustrates the pYA3467 plasmid.
20   Figure 11 illustrates the pYA3433 plasmid.
Figure 12 illustrates Coomassie staining of 12%
sodium dodecyl sulfate (SDS), polyacrylamide gel
electrophoresis (PAGE) to show expression of the
recombinant hybrid HBcAg-pre-S antigen in S. typhi ΔphoPQ
25   Δasd vaccine strains, wherein the arrow indicates the
position of the recombinant antigen for lane 1,
polypeptide SDS-PAGE size standards; lane 2, MGN-1191;
lane 3, MGN-1191/pYA3167, transformant #1 (χ8281); lane
4, MGN-1191/pYA3167, transformant #2; lane 5, MGN-
30   1191/pYA3167, transformant #3; lane 6, MGN-1256; lane 7,
MGN-1256/pYA3167, transformant #1 (χ8280); lane 8, MGN-

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1256/pYA3167, transformant #2; lane 9, MGN-1256/pYA3167, transformant #3; and lane 10, χ6212/pYA3167.

Figure 13 illustrates immunostaining with anti-HBV-preS monoclonal antibody following SDS-12% PAGE to show expression of the recombinant hybrid HBcAg-pre-S antigen in *S. typhi* ΔphoPQ Δasd vaccine strains, wherein the arrow indicates the position of the recombinant antigen for lane 1, polypeptide SDS-PAGE size standards; lane 2, MGN-1191; lane 3, MGN-1191/pYA3167, transformant #1 (χ8281); lane 4, MGN-1191/pYA3167, transformant #2; lane 5, MGN-1191/pYA3167, transformant #3; lane 6, MGN-1256; lane 7, MGN-1256/pYA3167, transformant #1 (χ8280); lane 8, MGN-1256/pYA3167, transformant #2; lane 9, MGN-1256/pYA3167, transformant #3; and lane 10, χ6212/pYA3167.

Figure 14 illustrates the pCMV beta-asd plasmid.

Description of the Preferred Embodiments

The present invention is based upon the discovery made in *S. typhimurium*, which is predictive for other Salmonella such as S. *typhi*, that *Salmonella* having a functional *rpoS* gene and an RpoS⁺ phenotype have a high immunogenicity and can be advantageously used as vaccines and as carrier microbes. Such vaccines and carrier microbes can serve as vehicles for delivering desired gene products such as antigens to humans as well as for delivering nucleic acids, either DNA or RNA, to target human cells.

The *rpoS* gene product contributes to the virulence of *Salmonella typhimurium* in mice, at least in part, by regulating expression of chromosomal gene determinants of virulence and is believed to contribute to *S. typhi*

virulence in humans through a similar mechanism. Much of
the work that has led to the development of live *S. typhi*
vaccines for immunization of humans has relied upon
studies using strains of *S. typhimurium* tested in mice.

5   These *S. typhimurium* strains cause an invasive infection
in susceptible mice that resembles typhoid in humans.
(Carter and Collins, *J. Exp. Med. 139*:1189-1203; Hohmann
et al., *Infect Immun 22*:763-770, 1978; Coynaut et al.
Molecular Microbiol. 22:149-160, 1996). Furthermore, the

10   role of the *rpoS* gene in the invasiveness and virulence
of *Salmonella typhimurium* is relevant to the invasiveness
and virulence of *Salmonella typhi* which lack a virulence
plasmid inasmuch as strains of *Salmonella typhimurium*
cured of the virulence plasmid have been shown to

15   colonize Peyers patches with efficiency similar to that
of the wild-type microorganisms (Gulig and Curtiss,
Infect Immun 55:2891-2901, 1987; Hackett et al., *J Infect
Dis 153*:1119-1125, 1986). The results of studies in
Salmonella typhimurium, which are thus also applicable to

20   *Salmonella typhi*, show that the *rpoS* gene product
controls the expression of chromosomally encoded genes
which are important for invasiveness and virulence.
(Nickerson and Curtiss, *Infect and Immun 65*:1814-1823,
1997; Kowarz et al, *J Bacteriol 176*:6852-6860, 1994).

25   In studies described in the Examples below, the
inventors herein found that the presence of a functional
rpoS gene is necessary for the early stages of the
Salmonella typhimurium infection process at the level of
the Peyer's patches and that the *rpoS* gene product acts

30   through an interaction with chromosomal genes. In
particular, it was discovered that an *rpoS* mutant of *S.
typhimurium* exhibited wild-type abilities to attach to

and invade cells of a human embryonic intestinal
epithelial cell line, Int-407, and a murine macrophage-
like cell line, J774. In addition, mutation in the *rpoS*
gene did not affect the intracellular survival of *S.*
typhimurium in either the J774 macrophage-like cells or
rat bone marrow-derived macrophages. However, the *rpoS*
mutant demonstrated a decreased ability to colonize
murine Peyer's patches after oral inoculation as compared
to its wild-type virulent parent strain.

        In addition, virulence plasmid-cured derivatives
of the *rpoS* mutant were recovered in lower numbers from
murine Peyer's patches than were plasmid-cured
derivatives of the isogenic wild-type *S. typhimurium*.
This indicates that RpoS regulation of chromosomally-
encoded genes is important for colonization of the murine
gut associated lymphoid tissue (GALT) by *S. typhimurium*.

        Microscopic analysis of histological sections
taken from Peyer's patches after peroral infection of
mice showed that, unlike its wild-type virulent parent
strain, the isogenic *rpoS* mutant did not destroy the
follicle-associated epithelium of the GALT. Furthermore,
the *rpoS* mutant demonstrated a decreased ability to
adhere to histological sections of murine Peyer's patches
as compared to its wild-type parent. These data
implicate the *rpoS* gene in the initial stages of systemic
infection by *Salmonella* involving interaction of
Salmonella with cells of the Peyer's patches.

        As a result of the decreased ability of *rpoS*
mutants to colonize Peyer's patches, earlier reports have
suggested that *Salmonella* strains having an inactivating
mutation in the *rpoS* gene are attractive candidates for
use in live oral attenuated vaccines. (Nickerson and

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Curtiss, *supra*, 1996). In contrast to this earlier work,
however, the present invention is directed to *Salmonella*
strains and other bacteria having a functional *rpoS*⁺ gene
along with an attenuating mutation in another gene. As a

5   result, the strains of the present invention are able to
colonize Peyer's patches, or similar tissues including,
for example, other lymphoid tissues of the GALT in
humans, without destroying the invaded cells in order to
achieve a high immunogenicity upon administration orally.

10 Furthermore, the M cells of the follicle-associated
lymphoid tissue of the GALT are functionally,
morphologically and structurally the same as the M cells
associated with other mucosal lymphoid tissues in the
body such as conjunctiva associated lymphoid tissue

15 (CALT), bronchus associated lymphoid tissue (BALT) and
nasal associated lymphoid tissue (NALT), as well as
lymphoid tissues in the rectum, etc. Thus, it is
believed that the presence of a functional *rpoS*⁺ gene in
the *Salmonella* will play an important role in the

20 invasion and colonization of these tissues when
administration is by routes including oral, intranasal,
rectal, etc. In fact, as shown in the examples below,
RpoS⁺ *S. typhimurium*, both non-recombinant and recombinant
expressing a foreign antigen, are superior to isogenic

25 RpoS⁻ *S. typhimurium* strains in conferring protective
immunity and in eliciting antibody responses to the
foreign antigens when delivered intranasally where
colonization of the NALT and BALT should be of prime
importance.

30   The *Salmonella* and other bacterial strains within
the scope of the present invention can be selected on the
basis of their having a functional *rpoS*⁺ gene which

produces a functional *rpoS* gene product. The *rpoS* gene
product is known to be a stationary-phase sigma factor
which is responsible for the control of a regulon of over
30 genes expressed in response to starvation and during
5   the transition to stationary phase. Protein products of
genes under the control of RpoS regulate a number of cell
functions including protection against DNA damage, the
determination of morphological changes, the mediation of
virulence, osmoprotection, and thermotolerance (Loewen
10   and Hengge-Aronis, *Annu. Rev. Microbiol. 48*:53-80, 1994).
Reference to RpoS phenotype herein is intended to mean
the manifestation of cell functions regulated by *rpoS*
gene expression in the microbe.

Many of the cell functions controlled by RpoS
15   regulation can be assessed in determining the RpoS
phenotype of a microbe. For example, one can analyze
cultures for catalase production. This test is based
upon RpoS positive regulation of the *katE* gene, which
produces hydroperoxidase II catalase. The culture medium
20   of strains carrying the wild-type *rpoS* allele grown to
stationary phase, bubble vigorously upon addition of
hydrogen peroxide, whereas minimal bubbling occurs in the
stationary phase culture medium of strains carrying a
mutant *rpoS* allele (Lowen, *J. Bacteriol. 157*:622-626,
25   1984; Mulvey et al., *Gene 73*:337-345, 1988). The RpoS
phenotypes of the attenuated *S. typhimurium* strains can
also be assayed by determining the sensitivity of these
strains to nutrient deprivation, acid or oxidative
stresses, and defective glycogen biosynthesis ability.
30   In a variation of this approach, the RpoS phenotype could
be determined by P22HT*int*-mediated transduction of the
rpoS allele into wild-type *S. typhimurium* χ3339, with

subsequent testing of the derived microbe for catalase
production as described above.

One can also genetically alter a strain which does
not contain a functional $rpoS^+$ gene using conjugation,

5   transformation, or transduction to introduce a functional
recombinant $rpoS^+$ gene which provides an RpoS$^+$ phenotype
in the catalase test. The recombinant $rpoS^+$ gene can be
from any suitable homologous or heterologous source,
preferably a homologous source.

10   It is also possible to introduce into *Salmonella*
containing a functional $rpoS^+$ gene another functional
recombinant $rpoS^+$ gene on a plasmid replicon or integrated
into the chromosome to further enhance the expression of
genes regulated by the RpoS protein. This might be

15   desirable in certain situations such as, for example, in
microbes having diminished *rpoS* gene expression, i.e.,
microbes which display nonoptimal colonization of the
GALT, or even in microbes in which the *rpoS* gene
expression is not diminished but a greater than normal

20   expression is desired.

It is also possible to provide a *Salmonella* or
other bacteria strain that is able to effectively
colonize the GALT or other lymphoid tissues even though
it does not express functional RpoS. For example, the

25   RpoS⁻ phenotype could be circumvented by incorporating
into an *rpoS* mutant strain at least one recombinant
virulence gene. Recombinant virulence gene or
recombinant RpoS virulence gene as referenced herein is
intended to mean that the recombinant gene is capable of

30   expressing a gene product having the same biological
function, i.e. facilitating effective colonization of the
GALT or other lymphoid tissue, as that of a chromosomal

virulence gene normally regulated by RpoS. However, expression of the incorporated recombinant virulence gene is controlled by regulatory elements that are not dependent upon the presence of functional RpoS, thereby

5   providing expression of the recombinant virulence gene product in the absence of functional RpoS. For example, a functional $rpoS^+$ gene is shown to be important for adherence by *Salmonella* to Peyer's patches, which is necessary for colonization of this tissue. One or more

10 genes responsible for this adherence is believed to be regulated by RpoS. One group of candidate genes controlling adherence to Peyer's patches that may be regulated by RpoS may be the *lpf* fimbrial operon (Bäumler et al., *Proc. Natl. Acad. Sci.*, USA 93:279-283, 1996).

15 Thus, the invasiveness and immunogenicity of an *rpoS* mutant microbe can be enhanced by transforming the microbe with one or more virulence genes under the control of regulatory elements that are not dependent upon the presence of functional RpoS.

20   In one embodiment of the present invention, the $rpoS^+$ bacteria strains, in particular $rpoS^+$ *Salmonella* strains, are attenuated derivatives of a pathogenic strain. By derivative or derived strain reference is made to a strain that has been genetically modified from

25 its parent from which it is descended. By pathogenic it is meant that the microbe is capable of causing disease or impairing normal physiological functioning. Reference to avirulence or attenuation herein, is intended to mean that a particular microbe strain is incapable of inducing

30 a full suite of symptoms of the disease state that is normally associated with its virulent non-attenuated pathogenic counterpart. Thus, avirulence or attenuation

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includes a state of diminished virulence or ability to
produce disease conditions and the attenuated or
avirulent microorganisms are not necessarily completely
absent of any ability to impair normal physiological
5   functioning of the host. In addition, an attenuated or
avirulent microbe is not necessarily incapable of ever
functioning as a pathogen, but the particular microbe
being used is attenuated with respect to the particular
individual being treated.

10   The *rpoS*⁺ strains of the present invention,
including *rpoS*⁺ *Salmonella* strains, are attenuated by
virtue of their containing an attenuating mutation in one
or more genes that renders the microorganism attenuated.
In a preferred embodiment, the strains have at least two
15   mutations each of which act to attenuate the
microorganism and which, in combination, significantly
increase the probability that the microorganism will not
revert to wild-type virulence. Mutations can be
insertions, partial or complete deletions or the like so
20   long as expression of the gene is diminished and
virulence is decreased. Attenuating mutations can be in
biosynthetic genes, regulatory genes and/or genes
involved in virulence. (See Doggett and Brown, *supra*).
Examples of mutations include, but are not limited to a
25   mutation in a *pab* gene, a *pur* gene, an *aro* gene, *asd*, a
dap gene, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*,
hemA, *cdt*, *cya*, *crp*, *phoP*, *phoQ*, *rfc*, *poxR*, *galU* and
combinations thereof. The skilled artisan will readily
appreciate that any suitable gene mutation can be used in
30   the present invention so long as the mutation of that
gene renders the microorganism attenuated.

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Methods are known in the art that can be used to generate mutations to produce the attenuated microbes of the present invention. For example, the transposon, Tn*10*, can be used to produce chromosomal deletions in a
5   wide variety of bacteria, including *Salmonella* (Kleckner et al., *J. Mol. Biol. 116*:125-159, 1977; EPO Pub. No. 315,682; U.S. Patent No. 5,387,744).

Recently, new methods have become available for producing specific deletions in genes. These methods
10 involve initially selecting a gene in which the deletion is to be generated. In one approach the gene can be selected from a genomic library obtained commercially or constructed using methods well known in the art (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed.,
15 1989, Cold Spring Harbor Press, Cold Spring Harbor, NY). Clones containing the gene are isolated from the genomic library by complementation of a strain which contains a mutation in the same gene. Alternatively, when the DNA sequence of the gene is known, selected primers for the
20 polymerase chain reaction method (PCR) can amplify the gene, often with some flanking sequence, from a sample of bacteria or from purified genomic DNA and the PCR product can be inserted into a cloning vector.

A specific deletion in the selected gene can be
25 generated by either of two general methods. The first method generates a mutation in a gene isolated from a population of clones contained in a genomic DNA library using restriction enzymes and the second method generates the mutation in a gene of known sequence using PCR.

30 Using the first method, the position of the gene on a vector is identified using transposon tagging and a restriction map of the recombinant DNA in the vector is

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generated. Information derived from the transposon
tagging allows all or a portion of a gene to be excised
from the vector using the known restriction enzyme sites.

The second method which is based upon PCR

5   methodology can be used when the DNA sequence of the gene
is known. According to this method, divergent PCR
primers amplify the upstream and downstream regions
flanking a specified segment of DNA to be deleted from
the gene and generate a PCR product consisting of the

10   cloning vector and upstream and downstream flanking
nucleotide sequences (Innes et al. Eds., PCR Protocols,
1990, Academic Press, New York). In a variation of this
method, PCR products are produced representing portions
of the gene or flanking sequence, which are then joined

15   together in a cloning vector.

The DNA containing the mutant gene can be
introduced into the bacterial host by transformation
using chemical means or electroporation, by recombinant
phage infection, or by conjugation. In preferred

20   embodiments the mutant gene is introduced into the
chromosomes of the bacteria which can be accomplished
using any of a number of methods well known in the art
such as, for example, methods using temperature-sensitive
replicons (Hamilton et al., *J. Bacteriol.* 171:4617-4622,

25   1989), linear transformation of *recBC* mutants (Jasin et
al., *J. Bacteriol.* 159:783-786, 1984), or host restricted
replicons known as suicide vectors (Miller et al., *J.
Bacteriol.* 170:2575-2583, 1988). The particular method
used is coupled with an appropriate counter selection

30   method such as, for example, fusaric acid resistance or
sucrose resistance followed by subsequent screening for
clones containing the mutant allele based upon phenotypic

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characteristics or by using PCR, nucleic acid
hybridization, or an immunological method.

The attenuated *rpoS*⁺ bacteria strains of the
present invention, in particular, attenuated *S. typhi*

5   mutants, can be used in the form of vaccines to deliver
recombinant antigens to a human or nucleic acids to
target cells of a human. Thus, it is apparent that the
present invention has wide applicability to the
development of effective recombinant vaccines against

10   bacterial, fungal, parasite or viral disease agents in
which local immunity is important and might be a first
line of defense. Some examples are recombinant vaccines
for the control of bubonic plague caused by *Yersinia
pestis*, of gonorrhea caused by *Neisseria gonorrhoea*, of

15   syphilis caused by *Treponema pallidum*, and of venereal
diseases as well as eye infections caused by *Chlamydia
trachomatis*. Species of *Streptococcus* from both group A
and group B, such as those species that cause sore throat
or heart disease, *Neisseria meningitidis, Mycoplasma*

20   *pneumoniae, Haemophilus influenzae, Bordetella pertussis,
Mycobacterium tuberculosis, Mycobacterium leprae,
Streptococcus pneumoniae, Brucella abortus, Vibrio
cholerae, Shigella* species, *Legionella pneumophila,
Borrelia burgdorferi, Rickettsia* species, *Pseudomonas*

25   *aeruginosa*, and pathogenic *E. coli* such as ETEC, EPEC,
UTEC, EHEC, and EIEC strains are additional examples of
microbes within the scope of this invention from which
genes could be obtained. Recombinant anti-viral
vaccines, such as those produced against influenza

30   viruses, are also encompassed by this invention.
Recombinant anti-viral vaccines can also be produced
against viruses, including RNA viruses such as

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Picornaviridae, Caliciviridae, Togaviridae, Flaviviridae,
Coronaviridae, Rhabdoviridae, Filoviridae,
Paramyxoviridae, Orthomyxoviridae, Bunyaviridae,
Arenaviridae, Reoviridae or Retroviridae; or DNA viruses

5   such as Hepadnaviridae, Paroviridae, Papovaviridae,
Adenoviridae, Herpesviridae or Poxviridae. Recombinant
vaccines to protect against infection by pathogenic
fungi, protozoa or parasites are also contemplated by
this invention.

10   Thus, in one set of embodiments, the present
invention can be described as a vaccine for the
immunization of a human comprising a live attenuated
derivative of a pathogenic bacteria such as a pathogenic
S. typhi wherein the derivative contains a functional

15   *rpoS* gene and expresses an RpoS$^+$ phenotype. The
attenuated bacteria is also capable of expressing a
recombinant gene derived from an organism that is a
pathogen of or that produces an allergen of the human.
           In embodiments in which the immunogenic component

20   of the vaccine is an allergen of the host, such a vaccine
can be used in an exposure regimen designed to
specifically desensitize an allergic host. Allergies to
pollens, mold spores, insect parts, animal dander and the
like are due to the inhalation of air and/or ingestion of

25   feed containing such allergens. The allergies that
result are associated with a presence of IgE antibodies
that bind to allergens which activate mast cells for
release of histamines. As is well known, desensitization
against allergens can be achieved by repetitive

30   parenteral immunization of extracts containing the
allergen. Likewise, it is known that oral ingestion of
raw honey containing pollens can be used to effectively

induce a state of tolerance against those allergens.
Oral ingestion with such allergens can on the one hand
induce an SIgA response that could block the ability of
allergens to react with IgE and mast cells or if

5   administered in sufficient quantity could serve to
suppress the synthesis of IgE antibodies, that is to
induce tolerance. Since the specific allergenic molecule
in many allergens has been identified and the cDNA cloned
to obtain the nucleotide sequence specifying the

10   allergen, it is now possible to genetically engineer
heterologous host cells to express the allergen (see for
example, Valenta et al, *Allergy 53*:552-561. 1998; Olsson
et al., *Clin. Exp. Allergy 28*:984-991. 1998; Soldatova et
al., *J. Allergy Clin. Immunol. 101*:691-698, 1998;

15   Asturias et al, *Clin Exp Allergy 27:1307-1313*; Twardosz
et al, *Biochem Biophys Res Commun 239*:197-204, 1997).
Accordingly, the attenuated RpoS⁺ *Salmonella* of the
present invention can be engineered to express an
allergen, possibly in a modified immunogenic but

20   nonallergenic form to induce a state of tolerance or to
actively promote the production of SIgA against the
allergen. The RpoS⁺ attenuated *Salmonella* described
herein have been shown to be effective in eliciting
immune responses and, hence, it follows that use of such

25   RpoS⁺ *Salmonella* to express modified allergens would be
likely to be effective in ameliorating the consequences
of exposure of humans to allergens by inhalation or
ingestion.

In other embodiments, the recombinant gene

30   expresses a gamete-specific antigen which is capable of
eliciting an immune response that confers an

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antifertility effect upon the immunized individual (See, U.S. Patent 5,656,488).

The attenuated microbes of this invention can additionally be used as vectors for the synthesis of

5   various host proteins. Because the attenuated microbes of this invention are able to traverse a variety of immunocompetent structures including gut-associated lymphoid tissue (GALT), mesenteric lymph nodes and spleen after introduction into the host, such microbes can be

10   used to target a variety of immunoregulatory products. Accordingly, one or more genes encoding immunoregulatory proteins or peptides can be recombinantly introduced into the attenuated microbes such that when the microbes taking up residence in the appropriate immunocompetent

15   tissue are capable of expressing the recombinant product to suppress, augment or modify the immune response in the host. Examples of immunoregulatory molecules include but are not limited to: colony stimulating factors (macrophage, granulocyte, or mixed), macrophage

20   chemotoxin, macrophage inhibition factor, leukocyte inhibitory factors, lymphotoxins, blastogenic factor, interferon, interleukins, tumor necrotizing factor, cytokines, and lymphokines.

The attenuated microbes of the present invention

25   are also contemplated for use to deliver and produce pharmacologically active products that might stimulate or suppress various physiological functions (i.e., growth rate, blood pressure, etc.). In such microbes, the recombinant gene encodes said pharmacologically active

30   products.

The recombinant gene of the microbes of the present invention can be incorporated into a "balanced-

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lethal" system which selects for microorganisms containing and capable of expressing the recombinant gene by linking the survival of the microorganism to the continued presence of the recombinant gene. "Balanced-

5   lethal" mutants of this type are characterized by a lack of a functioning native chromosomal gene encoding an enzyme which is essential for cell survival, preferably an enzyme which catalyzes a step in the biosynthesis of diaminopimelic acid (DAP) and even more preferably a gene

10 encoding beta aspartate semialdehyde dehydrogenase (Asd). DAP pathway enzymes and Asd are required for cell wall synthesis. The mutants also contain a first recombinant gene which can serve to complement the non-functioning chromosomal gene and this is structurally linked to a

15 second recombinant gene encoding the desired product. Loss of the complementing recombinant gene causes the cells to die by lysis when the cells are in an environment where DAP is lacking. This strategy is especially useful since DAP is not synthesized by

20 eukaryotes and, therefore, is not present in immunized host tissues. Methods of preparing these types of "balanced lethal" microbes are disclosed in U.S. Patent No. 5,672,345.

        By immunogenic agent is meant an agent used to

25 stimulate the immune system of an individual, so that one or more functions of the immune system are increased and directed towards the immunogenic agent. Immunogenic agents include vaccines. Immunogenic agents can be used in the production of antibodies, both isolated polyclonal

30 antibodies and monoclonal antibodies, using techniques known in the art.

30

An antigen or immunogen is intended to mean a molecule containing one or more epitopes that can stimulate a host immune system to make a secretory, humoral and/or cellular immune response specific to that
5   antigen.

An epitope can be a site on an antigen to which an antibody specific to that site binds. An epitope could comprise 3 amino acids in a spatial conformation which is unique to the epitope; generally, an epitope consists of
10   at least 5 amino acids and more usually, at least 8-10 amino acids. The term "epitope" is intended to be interchangeable with the term "antigenic determinant" as used herein. The term "epitope" is also intended to include T-helper cell epitopes in which an antigenic
15   determinant is recognized by T-helper cells through association with major histocompatibility complex class II molecules. In addition, the term epitope includes any antigen, epitope or antigenic determinant which is recognized by cytotoxic T cells when presented by a MHC
20   class I molecule on the surface of an antigen presenting cell. A cytotoxic T cell epitope can comprise an amino acid sequence of between about 6 to about 11 amino acids, and preferably comprises a sequence of 8 or 9 amino acids.

25   By vaccine is meant an agent used to stimulate the immune system of an individual so that protection is provided against an antigen not recognized as a self-antigen by the immune system. Immunization refers to the process of inducing a continuing high level of antibody
30   and/or cellular immune response in which T-lymphocytes can either kill the pathogen and/or activate other cells (e.g., phagocytes) to do so in an individual, which is

directed against a pathogen or antigen to which the
organism has been previously exposed. Although the
phrase "immune system" can encompass responses of
unicellular organisms to the presence of foreign bodies,
5   in this application the phrase is intended to refer to
the anatomical features and mechanisms by which an
individual produces antibodies against an antigenic
material which invades the cells of the individual or the
extra-cellular fluid of the individual and is also
10   intended to include cellular immune responses. In the
case of antibody production, the antibody so produced can
belong to any of the immunological classes, such as
immunoglobulins, A, D, E, G or M. Of particular interest
are vaccines which stimulate production of immunoglobulin
15   A (IgA) since this is the principle immunoglobulin
produced by the secretory system of warm-blooded animals,
although vaccines of the invention are not limited to
those which stimulate IgA production. For example,
vaccines of the nature described herein are likely to
20   produce a broad range of other immune responses in
addition to IgA formation, for example cellular and
humoral immunity. Immune responses to antigens are well
studied and widely reported. A survey of immunology is
provided in Elgert, Klaus D., *Immunology*, Wiley Liss,
25   Inc., (1996); Stites et al., *Basic & Clinical Immunology;*
7th Ed., Appleton & Lange, (1991) the entirety of which
are incorporated herein by reference.

An "individual" treated with a vaccine of the
present invention is defined herein as referring to a
30   human host.

Microbes as used herein can include bacteria,
protozoa and unicellular fungi. The term parasite as

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used herein is intended to include protozoans such as
species of *Plasmodium* and *Toxoplasma* as well as species
of *Entamoeba*, *Leishmania* and *Trypanosoma* and helminths
such as trematodes, cestodes and nematodes. Viruses as
5 used herein can include RNA viruses such as, for example,
Picornaviridae, Caliciviridae, Togaviridae, Flaviviridae,
Coronaviridae, Rhabdoviridae, Filoviridae,
Paramyxoviridae, Orthomyxoviridae, Bunyaviridae,
Arenaviridae, Reoviridae and Retroviridae; and DNA
10 viruses such, for example, as Hepadnaviridae,
Paroviridae, Papovaviridae, Adenoviridae, Herpesviridae
and Poxviridae.

Reference to a recombinant gene is intended to
mean genetic material that is transferred by human
15 intervention from a first organism into a second organism
which upon reproduction gives rise to descendants
containing the same genetic material. Generally, such
exchange of genetic material from the first organism to
the second organism either does not take place or rarely
20 takes place in nature.

The term gene as used herein in its broadest sense
represents any biological unit of heredity. It is not,
however, necessary that the recombinant gene be a
complete gene as is present in the parent organism and
25 capable of producing or regulating the production of a
macromolecule such as for example, a functioning
polypeptide. The recombinant gene may, thus, encode all
or part of an antigenic product. Furthermore, the
recombinant gene can also include DNA sequences that
30 serve as promoters, enhancers or terminators and DNA
sequences that encode repressors or activators that
regulate expression of a recombinant gene encoding all or

part of an antigen. A recombinant gene can also refer to
gene fusions which encode polypeptide fusion products.
The encoded gene product can, thus, be one that was not
found in that exact form in the parent organism. For

5   example, a functional gene coding for a polypeptide
antigen comprising 100 amino acid residues can be
transferred in part into a carrier microbe so that a
peptide comprising only 75, or even 10, amino acid
residues is produced by the cellular mechanisms of the

10 host cell. However, if this gene product can serve as an
antigen to cause formation of antibodies against a
similar antigen present in the parent organism or as a T-
cell epitope recognized by T-helper cells, the gene is
considered to be within the scope of the term gene as

15 defined in the present invention. Alternatively, if the
amino acid sequence of a particular antigen or fragment
thereof is known, it is possible to chemically synthesize
the DNA fragment or analog thereof by means of automated
gene synthesizers or the like and introduce said DNA

20 sequence into the appropriate expression vector. This
might be desirable in order to use codons that are
preferred codons for high level expression in *Salmonella*.
At the other end of the spectrum is a long section of DNA
coding for several gene products, one or all of which can

25 be antigenic. For example, such a long section of DNA
could encode 5 to 15 proteins necessary for the synthesis
of fimbrial antigens (fimbriae), which mediate adhesion
of pathogens to host cells (Bäumler et al., *supra*). The
induction of an immune response against fimbriae can

30 provide protection against the pathogen. Thus, a gene as
defined and claimed herein is any unit of heredity
capable of producing an antigen. The gene can be of

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chromosomal, plasmid, or viral origin. It is to be understood that the term gene as used herein further includes DNA molecules lacking introns such as, for example, is the case for cDNA molecules, so long as the

5 DNA sequence encodes the desired gene product. Further, the term gene includes within its meaning RNA molecules that serve as genes of RNA viruses or the complement of such RNA molecules wherein the RNA molecule or complement thereof can serve as an mRNA to be transcribed into a

10 viral protein which is immunogenic. The term gene as used herein also includes a DNA sequence specifying the viral strand that serves as an mRNA to be translated into the viral protein which is the immunogenic.

       In order for the gene to be effective in eliciting
15 an immune response, the gene must be expressed. Expression of a gene means that the information inherent in the structure of the gene (the sequence of DNA bases) is transformed into a physical product in the form of an RNA molecule, polypeptide or other biological molecule by

20 the biochemical mechanisms of the cell in which the gene is located. The biological molecule so produced is referenced as the gene product. The term gene product as used here refers to any biological product or products produced as a result of the biochemical reactions that

25 occur under the control of a gene. The gene product can be, for example, an RNA molecule, a peptide, or a product produced under the control of an enzyme or other molecule that is the initial product of the gene, i.e., a metabolic product. For example, a gene can first control

30 the synthesis of an RNA molecule which is translated by the action of ribosomes into an enzyme which controls the formation of glycans in the environment external to the

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original cell in which the gene was found. The RNA
molecule, the enzyme, and the glycan are all gene
products as the term is used here. Any of these as well
as many other types of gene products, such as

5   glycoproteins, glycolipids and polysaccharides, will act
as antigens if introduced into the immune system of an
individual. Protein gene products, including
glycoproteins and lipoproteins, are preferred gene
products for use as antigens in vaccines.

10   In order for a vaccine to be effective in
stimulating cellular immunity or in producing antibodies,
the antigenic materials must be released and/or presented
in such a way to trigger the induction of a cellular
immunity and/or induce the antibody-producing mechanism

15   of the vaccinated individual. Therefore, the microbe
carrier of the gene product must be introduced into the
individual. In order to stimulate a preferred response
of the gut-associated lymphoid tissue (GALT) or bronchus-
associated lymphoid tissue (BALT), introduction of the

20   microbe or gene product directly into the gut or bronchus
is preferred, such as by oral administration, gastric
intubation or intranasally in the form of aerosols,
although other methods of administering the vaccine, such
as intravenous, intramuscular, subcutaneous injection or

25   intramammary or intrapenial or vaginal or rectal
administration, are possible.

            The attenuated microbe can be used as a carrier
microbe, for example, for an antigen or for a DNA vaccine
vector, and once the carrier microbe is present in the

30   individual, the antigen needs to become available to the
individual's immune system. In the case of a carrier
microbe for delivery of a nucleic acid molecule, the

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nucleic acid molecule needs to be released within the
target cell. This can be accomplished when the carrier
microbe dies so that the antigen molecules or nucleic
acid molecules are released. Of course, the use of
5 "leaky" attenuated mutants that release the contents of
the periplasm without lysis is also possible.

Alternatively, a gene can be selected that
controls the production of an antigen that will be made
available by the carrier cell to the outside environment
10 prior to the death of the cell. In this way, it is
possible to use a viable microbe that will persist in the
vaccinated individual, for example in its Peyer's patches
or other GALT, NALT or BALT, etc., and continue to
produce antigen, thereby continually inducing antibody
15 formation and/or a cellular immune response. A preferred
gene product under these circumstances is a product that
is transferred through the cell membrane of the
attenuated carrier microbe into the external environment
or a product that becomes attached to or embedded in the
20 external membrane so that all or part of the gene product
is exposed to the environment. Typical of this latter
type of gene product are antigens normally found on the
surface of the organism against which protection is
desired. If these antigens are transported to the
25 bacterial cell surface in a normal manner, antibody
formation against the antigens will be enhanced.

Nucleic acid vaccines are well known in the art
(see e.g., Ulmer et al., Amer. Soc. Microbiol. News
62:476-479, 1996; Ulmer et al., Curr. Opinion. Immunol.
30 8:531-536, 1996; and Robinson, H.L., Vaccine 15:785-787,
1997) and delivery of DNA vaccines by attenuated bacteria
with subsequent stimulation of an immune response against

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the protein-encoded by the DNA vaccine has been described
(Sizemore et al., Vaccine 15:804-806, 1997). Thus, it is
expected that the attenuated microbes of the present
invention can also be used as delivery vehicles for DNA

5   vaccines. Typically, bacteria containing such DNA
vaccines do not themselves express the gene product
encoded by the DNA vaccine, but release the DNA vaccine
into one or more human tissues, where the gene product is
then expressed by host cell transcription and translation

10 machinery. However, it is also contemplated that a DNA
vaccine for immunization against RNA viruses can be
constructed in which copies of the RNA viral genome, or
of a protein-encoding portion thereof, will be made in
the cytoplasm of the attenuated bacteria. Such RNA

15 molecules would be released into the human tissues, e.g.,
by lysis of the attenuated bacteria, where they would
serve as mRNA for synthesis of immunogenic viral
protein(s).

The use of pathogens to deliver antigens from

20 other pathogens to the GALT or BALT would be
inappropriate if it were not for the fact that such
pathogens can be rendered attenuated while retaining
ability to colonize these tissues.

The organism from which the recombinant gene is

25 derived can be any human pathogen or may be an organism
that produces an allergen or other antigen to which a
human can be sensitive. Allergens are substances that
cause allergic reaction, in this case in the human which
will be vaccinated against them. Many different

30 materials can be allergens, such as animal dander and
pollen, and the allergic reaction of individuals will
vary for any particular allergen. It is possible to

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induce tolerance to an allergen in an individual that
normally shows an allergic response. The methods of
inducing tolerance are well-known and generally comprise
administering the allergen to the individual in

5   increasing dosages. Further discussion of tolerance
induction is given in the Barrett textbook previously
cited. Lastly, the host individual itself can serve as a
source of genetic material when immunoregulatory genes or
genes for other pharmacologically active substances are

10 being expressed by the vectors.

        Administration of a live vaccine of the type
disclosed above to an individual can be by any known or
standard technique. These include oral ingestion,
gastric intubation, or broncho-nasal-ocular spraying.

15 All of these methods allow the live vaccine to easily
reach the NALT, GALT or BALT cells and induce antibody
formation and cell mediated immunity and are the
preferred methods of administration. Other methods of
administration, such as intravenous injection, that allow

20 the carrier microbe to reach the individual's blood
stream can be acceptable. Intravenous, intramuscular or
intramammary injection are also acceptable with other
embodiments of the invention, as is described later.

        Any of a number of commonly used recombinant DNA

25 techniques can be used in producing the attenuated
microbes of the present invention which are capable of
expressing a recombinant gene. Following ligation to a
plasmid, phage or cosmid vector the recombinant molecules
so formed can be transferred into a host cell by various

30 means such as conjugation, or transformation (uptake of
naked DNA from the external environment, which can be
artificially induced by the presence of various chemical

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agents, such as calcium ions), including electroporation.
Other methods such as transduction are also suitable,
wherein the recombinant DNA is packaged within a phage
such as transducing phage or cosmid vectors. Once the
5   recombinant DNA is in the carrier cell, it may continue
to exist as a separate autonomous replicon or it may
insert into the host cell chromosome and be reproduced
along with the chromosome during cell division.

        Once the genetic material has been transferred,
10   the microbes containing the transferred genetic material
are selected.

        The immunization dosages required will vary with
the antigenicity of the gene product and need only be an
amount sufficient to induce an immune response. Routine
15   experimentation will easily establish the required
amount. Multiple dosages are used as needed to provide
the desired level of protection.

        The pharmaceutical carrier or excipient in which
the vaccine is suspended or dissolved may be any solvent
20   or solid or encapsulating material such as for a
lypholized form of the vaccine. The carrier is non-toxic
to the inoculated individual and compatible with the
microorganism or antigenic gene product. Suitable
pharmaceutical carriers are known in the art and, for
25   example, include liquid carriers, such as normal saline
and other non-toxic salts at or near physiological
concentrations, and solid carriers, such as talc or
sucrose. Gelatin capsules can serve as carriers for
lypholized vaccines. Adjuvants may be added to enhance
30   the antigenicity if desired. When used for administering
via the bronchial tubes, the vaccine is preferably
presented in the form of an aerosol. Suitable

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pharmaceutical carriers and adjuvants and the preparation

of dosage forms are described in, for example,

Remington's Pharmaceutical Sciences, 17th Edition,

(Gennaro, Ed., Mack Publishing Co., Easton, Pa.; 1985).

5   Immunization of an individual with a pathogen-

derived gene product can also be used in conjunction with

prior immunization with the attenuated derivative of a

pathogenic microorganism acting as a carrier to express

the gene product specified by a recombinant gene from a

10   pathogen. Such parenteral immunization can serve as a

booster to enhance expression of the secretory immune

response once the secretory immune system to that

pathogen-derived gene product has been primed by

immunization with the carrier microbe expressing the

15   pathogen-derived gene product to stimulate the lymphoid

cells of the GALT or BALT. The enhanced response is

known as a secondary, booster, or anamnestic response and

results in prolonged immune protection of the host.

Booster immunizations may be repeated numerous times with

20   beneficial results.

In another embodiment, the present invention

provides a method for assessing the immunogenicity of a

Salmonella comprising determining the RpoS phenotype of

the *Salmonella*. The method is also applicable for other

25   bacteria such as *Shigella* and *E.coli* and *Salmonella-*

Shigella hybrids, Salmonella-E.coli hybrids and *Shigella-*

E.coli hybrids. The presence of an RpoS$^+$ phenotype

confers upon the microbe the ability to invade and

colonize the lymphoid tissue associated with the

30   particular route of administration used such as, for

example, the GALT following oral administration or other

lymphoid tissues, such as the NALT or BALT following

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other routes of administration. This in turn results in
a high level of immunogenicity. Thus, detecting the
presence of an RpoS$^+$ phenotype indicates that the microbe
will have a high level of immunogenicity compared to a
5   microbe that is RpoS$^-$, but otherwise genetically
identical.

The RpoS$^+$ phenotype can be assessed by determining
the properties of the microbe. This can be done by any
of a number of possible methods. For example, by
10   analyzing cultures for catalase production. This test is
based upon RpoS positive regulation of the *katE* gene,
which produces hydroperoxidase II catalase. The culture
medium of strains carrying the wild-type *rpoS* allele
bubble vigorously upon addition of hydrogen peroxide,
15   whereas minimal bubbling occurs in the culture medium of
strains carrying a mutant *rpoS* allele (Lowen, *J.
Bacteriol. 157*:622-626, 1984; Mulvey et al., *Gene 73*:337-
345, 1988). Other methods can also be used for
determining the RpoS phenotypes of the attenuated
20   *Salmonella* or other bacteria strains including
determining the sensitivity of the strains to nutrient
deprivation, acid or oxidative stresses, and defective
glycogen biosynthesis ability. In a variation of this
approach, the rpoS allele can be transduced into a wild-
25   type *Salmonella* and the resultant derivative *Salmonella*
tested for RpoS phenotype.

One can also assess the RpoS$^+$ phenotype by
determining the genetic make up of the microbe wherein
the presence of a functional *rpoS$^+$* gene capable of
30   producing a function *rpoS* gene product indicates an RpoS$^+$
phenotype.

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Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the

5   specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

10

General Methods:

     The bacterial strains used in the present studies were constructed using the following general materials and methods. Listings of phages, plasmids and micro-

15   organisms used in constructing the strains are given in Tables 1 and 2.

Table 1. Microorganisms

Strain Designation	Relevant Genotype	Source/Reference/Derivation
Salmonella typhi Strains		
χ3743	ISP1804 Type 46	1983 isolate from Chilean patient, received from D. Hone, Center for Vaccine Development, MD
χ3744	ISP1820 cys trp	ATCC 55116; 1983 isolate from Chilean patient; received from D. Hone
χ3745	ISP2822 Type E1	ATCC 55114; 1983 isolate from Chilean patient; received from D. Hone
χ3746	ISP2825 Type E1	1983 isolate from Chilean patient; received from D. Hone
χ3769	Ty2 Type E1 rpoS cys	Louis Baron, Walter Reed Army Institute of Research
χ3927	Ty2 Δcrp-11 Δ[zhb::Tn10] Δcya-12 Δ[zid-62::Tn10]	ATCC 55117
χ4073	Ty2 Δ[crp-cdt]-10 Δ[zhb::Tn10] Δcya-12 Δ[zid-62::Tn10]	ATCC 55118
χ8203	cys trp	ATCC 9992V; AMC strain Boxhill 58V
χ8204	cys trp	ATCC 33458; CDC 2862-79
χ8205	Ty21a galE rpoS cys trp	ATCC 33459; CDC 2861-79
χ8206	cys trp aroA serC purA155	ATCC 39926; Stanford 531Ty; derivative of CDC10-80
χ8207	cys trp	ATCC 10749; AMC 42-A-63
χ8208	Ty2 cys	ATCC 19430; NCTC 8385

Table 1 (Continued)

Strain	Genotype	Source
χ8209	cys trp	ATCC 9993; AMC 42-A-63
MGN-1018	Ty2 rpoS cys ΔphoPQ23	MEGAN Health Inc., St. Louis, MO
MGN-1038	ISP1820 cys trp ΔphoPQ23	MEGAN Health, Inc., St. Louis, MO
MGN-1191	ISP1820 cys trp ΔphoPQ23 ΔasdA16	MEGAN Health, Inc., St. Louis, MO
MGN-1256	Ty2 rpoS cys ΔphoPQ23 ΔasdA16	MEGAN Health, Inc., St. Louis, MO
χ8280	MGN-1256(pYA3167)	
χ8281	MGN-1191(pYA3167)	
χ8434	Ty2 rpoS+ cys ΔphoPQ23	MGN-1018 by introducing recombinant rpoS+ gene from pYA3467
χ8435	Ty2 rpoS+ cys ΔphoPQ23 ΔasdA16 (pYA3167)	χ8280 by introducing recombinant rpoS+ gene from pYA3467
χ8438	Ty2 Type E1 rpoS+ cys (RpoS+)	χ3769 by introducing recombinant rpoS+ gene from pYA3467

Salmonella typhimurium Strains

Strain	Genotype	Source
χ3000	LT2-2 prototroph	Received from C. Turnbough
χ3181	SR-11 pStSR100+ wild type	Isolated by passage from murine Peyer's patch. Gulig and Curtiss, Infect. Immun. 65:2891-2901 (1987).
χ3339	SL1344 pStSL100+ hisG rpsL, colicin+	Animal passaged isolate of SL1344, isolated from liver of moribund mouse after p.o. infection. Gulig and Curtiss, Infect. Immun. 65:2891-2901 (1987).

Table 1 (Continued)

χ3340	SL1344 pStSL100⁻ hisG rpsL, colicin⁺	Virulence plasmid-cured derivative of χ3339; Gulig and Curtiss, *Infect. Immun.* 65:2891-2901 (1987).
χ3420	SL1344 hisG rpsL xyl fli-8007:: Tn10	P22HTint(χ3376)→χ3339 with selection for Tc^R Mot^- Fla^-.
χ3422	SR-11 fli-8007::Tn10	P22HTint(χ3376)→χ3181 with selection for Tc^R Mot^- Fla^-
χ3679	SR-11 ΔaroA554	P22HTint(χ3678)→ χ3181 selecting Tc^r and screening for Aro^- followed by selection for tetracycline sensitivity, Aro^-.
χ3761	UK-1 wild-type prototroph	ATCC 68169; spleenic isolate from infected chick.
χ4973	SL1344 pStSL100⁺ hisG rpsL rpoS::RR10, colicin⁺	Nickerson and Curtiss, *Infect. Immun.*, 65:1814-1823 (1997)
χ8125	SL1344 pStSL100⁻ hisG rpsL rpoS::RR10, colicin⁺	Nickerson and Curtiss, *Infect. Immun.*, 65:1814-1823 (1997)
χ8214	UK-1 rpoS::RR10 Δcya-27 Δcrp-27	P22HTint(SF1005)→MGN-431 with selection for ampicillin resistance
χ8215	SR-11 rpoS::RR10 ΔaroA554	P22HTint(SF1005)→χ3679 with selection for ampicillin resistance

Table 1 (Continued)

χ8217	UK-1 rpoS::RR10 Δcya-27	P22HTint(SF1005)→MGN-232 with selection for ampicillin resistance
χ8296	SL1344 pstSL100⁺ Δcya-28 Δcrp-27 ΔasdA16 (RpoS⁺)	MEGAN Health, Inc.; χ3339 derivative with three defined deletion mutations
χ8309	SL1344 pstSL100t Δcya-28 Δcrp-27 ΔasdA16 rpoS (RpoS⁻)	P22HTint(χ4973)→ χ8296
MGN-232	UK-1 Δcya-27	MEGAN Health, Inc.; defined cya deletion derivative of χ3761
MGN-431	UK-1 Δcya-27 Δcrp-27	MEGAN Health, Inc.; defined crp deletion derivative of MGN-232
ATCC 14028s	prototroph, Tet^s	wild-type invasive strain obtained from F. Heffron
SF1005	14028s rpoS::RR10	F. Fang, Univ. Colorado Health Sci. Center

Table 1 (Continued).

E. coli Strains		
χ6101	K-12 DH5α F⁻ Ø80d lacZ ΔM15 Δ(lacZYA-argF)-4169 gln44 λ⁻ gyrA recA1 relA1 endA1 hsdR17 (r$_k$⁻, m$_k$⁺)	Guy Cardineau, Sungene Technologies, Inc., San Jose, CA
χ6212	K-12 F⁻ Ø80d lacZ ΔM15 Δ(lacZYA-argF)-4169 supE44 λ⁻ gyrA recA1 relA1 endA1 ΔasdA4 Δ[zhf-2::Tn10] hsdR17 (r$_k$⁻, m$_k$⁺)	This lab
MGN-026	K-12 F⁻ Ø80d lacZ ΔM15 Δ(lacZYA-argF)-4169 supE44 λpir gyrA recA1 relA1 endA1 hsdR17 (r$_k$⁻, m$_k$⁺)	λpir→χ6101; MEGAN Health, Inc., St. Louis, MO
MGN-617	thi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA RP4-2-Tc::Mu λpir, ΔasdA4 Δ[zhf-2::Tn10]	MEGAN Health, Inc., St. Louis, MO

Table 2. Phages and Plasmids

Bacteriophage	Description	Source/Reference
P22HTint	high frequency generalized transducing mutant of the temperate lambdoid phage P22	Schmeiger, *Mol. Gen. Genet.* 119:75-88, 1972; Jackson et al., *J. Mol. Biol.*154:551-563, 1982; Ray et al., *Mol. Gen.Genet.* 135:175-184, 1974.
P22 H5	clear plaque forming mutant of P22HTint	Casjens et al., *J. Mol. Biol.* 194:411-422, 1987.
Plasmids		
pSK::*rpoS*	*S. typhimurium* 14028 *rpoS*+ gene cloned into the *EcoRV* site of pBlueScript/SK	F. Fang, Univ. Colorado Health Sci. Center
pMEG-003	*pir*-dependent R6K *ori* Tc^r *asd$^+$*	MEGAN Health, Inc., St. Louis, MO
pMEG-006	*pir*-dependent R6K *ori* Tc^r Δ*asdA16*	Megan Health, Inc., St. Louis, MO
pMEG-068	Contains *phoQ* gene	MEGAN Health, Inc., St. Louis, MO
pMEG-149	Amp^R mobilizable *pir*-dependent suicide vector; containing the *sacBR* genes from *B. subtillis*, RK2 *mob*, R6K *ori*	MEGAN Health, Inc., St. Louis, MO
pMEG-210	*phoQ* deletion of pMEG-068	MEGAN Health, Inc., St. Louis, MO

Table 2 (Continued)

pMEG-213	Derivative of pMEG-149 containing phoPQ23 defined deletion of pMEG-210	MEGAN Health, Inc., St. Louis, MO
pMEG-328	Derivative of pNEB-193 containing the *S. typhimurium* UK-1 *rpos⁺* gene cloned into the *Bam* HI and *XbaI* sites	MEGAN Health, Inc., St. Louis, MO
pMEG-375	*cat* gene from pACYC184 cloned into pMEG-149	MEGAN Health, Inc., St. Louis, MO
pNEB-193	pUC19 derivative that carries single restriction sites for unique 8bp cutters *AscI*, *PacI* and *PmeI* within the polylinker region	New England Biolabs
pYA3167	*asd* – complementing plasmid; expresses the Hepatitis B virus (HBV) nucleocapsid pre-S1 and pre-S2 epitopes on HBV core	Nardelli-Haefliger et al., *Infect. Immun. 64*:5219-5224, 1996
pYA3433	contains *rpos⁺* gene cloned from pSK::*rpoS* into *SmaI* site of pMEG-149	This lab

Table 2 (Continued)

pYA3467	contains *S. typhimurium* This lab UK-1 *rpoS*⁺ gene from pMEG-328 cloned into *Pme*I and *Sma* I sites of pMEG-375

Bacterial strains were maintained as duplicate -70°C
frozen cultures suspended in 1% Bacto-peptone (Difco)
containing 5% glycerol and were also stored at -20°C in 1%
Bacto-peptone and 50% glycerol for routine use. Bacteria
5 were generally cultured in L Broth (Lennox, *Virology*
1:190-206, 1965) or Luria Broth (Luria et al., *J.*
Bacteriol. 74:461-476, 1957). Agar plates contained 1.5
% Difco Agar. Carbohydrate utilization was evaluated by
supplementing MacConkey (Difco) or Eosin Methylene Blue
10 agar base (Curtiss, *Genetics 58*:9-54, 1968) with 1% final
concentration of an appropriate carbohydrate. Minimal
liquid (ML) and minimal agar (MA) were prepared as
described in Curtiss (*J. Bacteriol. 89*:28-40, 1965) and
supplemented with nutrients at optimal levels. Buffered
15 saline with gelatin (BSG) was used routinely as a diluent
(Curtiss, 1965 supra).

Bacteriophage P22HT*int* was used for transduction
using standard methods (Davis et al., A Manual for
Genetic Engineering - Advanced Bacterial Genetics, Cold
20 Spring Harbor Laboratory Press, Cold Spring Harbor, New
York, 1979). An overnight culture of a donor strain was
diluted 1:20 into prewarmed Luria broth, grown for one
hour with shaking at 37°C, and then infected with P22HT*int*
at a multiplicity of infection (MOI) of 0.01. The
25 infection mixture was shaken overnight or for
approximately fifteen hours. A few drops of chloroform
were added to ensure complete bacterial cell lysis, and
the mixture was allowed to shake an additional ten
minutes at 37°C, then centrifuged at 7,000 rpm in a
30 Sorvall SS-34 rotor for ten minutes to remove bacterial
debris. The supernatant fluid was extracted and removed
to a clean tube with a drop or two of fresh chloroform

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and stored at 4°C. This method generally provides a phage
lysate containing about 10^{10} PFU/ml titered on χ3000.
Tetracycline was used in plates at 12.5 µg/ml to select
for Tn10 transductants, Tn10-induced mutations, or

5   merodiploid strains expressing the Tn10-derived
tetracycline-resistance genes from a chromosomally
integrated suicide vector. The Tn10 transposon excises
from the chromosome at a low frequency, often deleting a
portion of the genome flanking the transposon. Cells

10 which undergo an excision event also become sensitive to
tetracycline, and can be identified by plating on media
containing fusaric acid, which kills tetracycline-
resistant bacteria (Maloy and Nunn, *J. Bacteriol.*
145:1110-1112, 1981). Tetracycline-sensitive strains

15 which have lost an integrated suicide plasmid along with
the plasmid linked tetracycline-resistance genes can also
be selected on fusaric acid media.

        Tetracycline-resistant cultures were grown standing
overnight in L broth containing 12.5 µg/ml tetracycline

20 at 37°C to approximately 5×10^8 CFU/ml. These cultures
were diluted 1:40 into prewarmed L broth without
tetracycline and aerated at 37°C to a titer of about $2 \times$
10^9 CFU/ml, serially diluted into BSG, and plated from
these dilutions onto fusaric acid media. Fusaric acid

25 resistant colonies were selected after incubation for 48
hours at 37°C. Fusaric acid resistant isolates were
restreaked to fusaric acid media, then patched to
Penassay agar (Difco) with and without tetracycline to
confirm the loss of the Tn10-derived antibiotic

30 resistance element. Other phenotypes were scored where
indicated using appropriate media.

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Suicide vectors containing an ampicillin-resistance gene, a sucrose-utilization cassette, and an *incP* mobilization site were constructed. Mutant genes which have been introduced into these plasmids can be

5 introduced into the bacterial chromosome after transformation, or preferably by conjugation, to generate ampicillin-resistant (100 μg/ml) merodiploids. Such merodiploids can be grown on media containing 5% sucrose to select for the loss of the integrated plasmid along

10 with the ampicillin-resistance and sucrose-utilization genes. Ampicillin-sensitive strains can be phenotypically characterized for the presence of appropriate defined deletion mutant alleles.

Improved selection of merodiploids can be achieved

15 by introducing a *cat* gene conferring resistance to chloramphenical (40μg/ml) in addition to an ampicillin-resistance gene on the suicide vector. After merodiploid formation, selection on media with 5% succrose leads to loss of both drug-resistance genes and the succrose-

20 utilization genes. these recombinants can then be screened for the desired allele replacement.

Example 1

This example illustrates the role of the *rpoS* gene in efficient invasion and colonization of the GALT by *S.*

25 *typhimurium* using an *rpoS* mutant strain, χ4973, compared to its wild-type parent, χ3339.

Strain Construction:

χ3339 is a wild-type, virulent, animal-passaged isolate of *S. typhimurium* strain SL1344 described in

30 Gulig et al. (*Infect Immun 55*:2891-2901, 1987). SF1005 is an *rpoS*::RR10 mutant derived from *S. typhimurium* strain ATCC 14028s and containing an ampicillin

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resistance gene linked to the *rpoS*::RR10 mutant allele
(Fang et al., *Proc. Nat'l. Acad. Sci., USA 89*:11978-
11982, 1992). The mutant *rpoS*::RR10 allele was moved
into χ3339 using a P22HT*int* transducing phage lysate

5   prepared on SF1005 and selecting for ampicillin
resistance (Ap^r) due to the presence of the ß-lactamase
gene (*bla*) linked to the RR10 insertion in the *rpoS* gene.
The allelic exchange between SF1005 and χ3339 was
confirmed by Southern blot analysis, and the resulting

10   χ3339 *rpoS*::RR10 mutant derivative was designated as
χ4973. Transductants were screened for sensitivity to
P22HT*int* by cross streaking with P22H5, a clear plaque
mutant. Pseudolysogenic colonies were distinguished from
non-lysogens on Evans blue and uranine (EBU) indicator

15   agar (Sternberg et al., *Meth. Enzymol. 204*:2-43, 1991).
Media were supplemented with 50 µg ampicillin per ml when
required to select for χ4973.

     The presence of smooth lipopolysaccharide (LPS) in
χ4973 was confirmed using the method of Hitchcock et al.

20   (*J. Bacteriol. 154*:269-277, 1983). LPS was silver
stained by the method of Tsai et al. (*Anal Biochem
119*:115-119, 1982). This experiment showed that the
mutation in *rpoS* did not affect LPS structure.

Virulence of an RpoS Mutant in Mice:

25   The virulence of χ3339 was compared to that of the
rpoS mutant strain χ4973 upon oral inoculation of eight-
to ten-week old female BALB/c mice. Animal inoculation
for the determination of the fifty per cent lethal dose
(LD$_{50}$) was performed as described earlier with minor

30   modifications (Gulig et al., *Infect Immun 55*:2891-2901,
1987). Mice were deprived of food and water for four to

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six hours prior to peroral inoculation. Gastric acidity
was not neutralized prior to infection. LD_{50} titers were
determined according to the method of Reed and Muench
(*Am. J. Hyg.* 27:493-497, 1938) for each strain using
results obtained from four mice per inoculum dose
evaluated for a period of thirty days.

The peroral LD_{50} for the *rpoS* mutant strain χ4973 was
greater than 8 X 10^9 colony forming units per dose. This
value represented more than a four log increase over the
oral lethal dose of 8 X 10^5 colony forming units observed
for the wild-type parent strain χ3339. This result is
consistent with Fang et al., *supra*, who reported a three
log increase in the oral LD_{50} dose for SF1005, as compared
to the *rpoS*[+] parent strain ATCC 14028s. Further studies
were then conducted to determine why the *rpoS* mutant
strain was attenuated compared to its wild-type parent.

Comparative Testing of Attachment, Invasion and Survival:

Human embryonic intestinal epithelial cell line Int-
407 (Henle et al., *J. Immunol.* 79:54-59, 1957) and murine
macrophage-like cell line J774 (Ralph et al., *Nature*
257:393-394, 1975) were used to examine the effect of
rpoS on the adherence and invasive abilities of *S.
typhimurium.* Each cell line was maintained in Minimal
Essential Medium (MEM; GibcoBRL, Grand Island, NY)
containing Hank's Balance Salt Solution (HBSS; GibcoBRL),
2 mM glutamine, and 10% fetal calf serum (FCS; HyClone,
Logan, UT) at 37°C in an atmosphere containing 5% CO_2.
Cells were passaged every two to three days with medium
changes. Macrophage monolayers used in an infection
assay were prepared by gently scraping passaged cells
into solution, diluting the cell suspension, inoculating
wells of a 24-well microtiter dish, and incubating at 37°C

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in a 5% CO$_2$ environment. Int-407 cells were distributed
in a similar fashion, but were trypsinized for removal
from monolayers.

   Bacterial attachment and invasion assays using cells
5   from the human intestinal epithelial cell line, Int-407,
and the mouse macrophage-like cell line, J774, followed
methods according to Galan et al., *Proc Natl Acad Sci,
USA 86*:6383-6387, 1989, with minor modifications.
Bacteria were grown as static cultures in L broth at 37°C
10   to mid log phase or about 0.5 optical density as measured
at 600 nm. Because expression of *rpoS* and RpoS-regulated
genes increases as cells enter into stationary phase, a
control culture was also grown statically for four days
to saturation in order to establish the maximal level of
15   *rpoS* expression. Bacterial cultures were washed and
resuspended in HBSS immediately prior to infection of
monolayers. Int-407 monolayer attachment and invasion
was allowed to proceed for two hours at 37°C in MEM in an
atmosphere of 5% CO$_2$ and at an MOI of between two and ten
20   bacterial cells per Int-407 cell. Attachment and
invasion assays using J774 cells were performed as with
Int-407 cells, except that only one hour was allowed for
adherence and invasion. As a control for distinguishing
adhesion from phagocytosis of bacterial cells by the
25   monolayer cells, J774 cells were monitored at 4°C in the
presence of bacteria according to the method of Lee et
al., *Proc. Nat'l. Acad. Sci., USA 87*:4304-4308, 1990.

   Infected monolayers were washed three times with
isotonic phosphate-buffered saline (PBS) after the
30   attachment and invasion incubation, and then lysed with
PBS containing 0.1% sodium deoxycholate to assess the
total number of bacteria associated with the cultured

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cells. Duplicate monolayers infected in parallel were
incubated an additional two hours with MEM containing 10
µg/ml gentamicin in order to kill extracellular bacteria
prior to lysis so that the number of internalized
5 bacteria could be enumerated. Viable bacterial cell
counts were obtained by plating dilutions of lysed
monolayers onto L agar and incubating at 37°C for eighteen
to twenty four hours. Results are shown in Tables 3 and
4 below.

10

Table 3. Effect of an *rpoS*::RR10 mutation on adherence
to and invasion of Int-407 cells by *S.*
15 *typhimurium* χ3339 and its *rpoS* mutant
derivative, χ4973[a]

Strain	Growth phase	Percent adhesion[b]	Percent invasion[c]
χ3339	Exponential	59.2 ± 0.3	83.0 ± 26.9
χ4973	Exponential	51.4 ± 0.2	88.0 ± 22.0
χ3339	Stationary	12.8 ± 2.3	25.0 ± 2.4
χ4973	Stationary	34.0 ± 18.0	50.0 ± 4.0

a   The data are given as the means ± SEM for three
20   trials.

b   Percent of inoculum adherent to cells after
     incubation for 2 hours.

25 c   Percent of inoculum recovered after incubation for 2
     additional hours in gentamicin [10µg/ml].

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Table 4.   Effect of an *rpoS*::RR10 mutation on the
            adherence to and invasion of J774 cells by *S.
            typhimurium* χ3339 and its *rpoS* mutant
            derivative, χ4973[a]

Strain	Growth phase	Percent adhesion[b]	Percent invasion[c]
χ3339	Exponential	55 ± 4.1	66 ± 3.7
χ4973	Exponential	57 ± 1.5	46 ± 4.4
χ3339	Stationary	44 ± 3.2	14 ± 3.5
χ4973	Stationary	19 ± 2.4	11 ± 0.3

a   The data are given as the means ± SEM for three
     trials.

b   Percent of inoculum adherent to cells after
     incubation for 1 hour.

c   Percent of inoculum recovered after incubation for 2
     additional hours in gentamicin [10μg/ml].

When the *S. typhimurium* strains were grown to
exponential phase, the *rpoS*::RR10 mutant, χ4973, attached
to Int-407 and J774 cells to the same extent as its wild-
type parent, χ3339 (Tables 3 and 4). Percent invasion
was also the same for both strains in the intestinal
epithelial cell line, Int-407 (Table 3). However,
invasion showed a small decrease with the *rpoS*::RR10
mutant in the macrophage cell line, J774, compared to the
wild-type parent (Table 4). These data indicate that
when the *S. typhimurium* were in the exponential growth
phase, the *rpoS* gene contributed little or nothing to the
ability of the bacteria to attach to or invade into the
cells. When the bacterial cells were in the stationary
phase, however, results were equivocal. Whereas adhesion
and invasion were slightly increased with *rpoS*::RR10
mutants grown to stationary phase in Int-407 cells,

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adhesion was slightly decreased for the *rpoS*::RR10
mutants grown to stationary phase in J774 cells (Tables 3
and 4, respectively). In additional studies not shown,
no difference was observed in the ability of these
5   strains to adhere to or invade into J774 cells when
assays were conducted at 4°C (data not shown). These data
indicate that the *rpoS* gene product has little or no
effect on *in vitro* attachment to or invasion of
intestinal epithelial cells and macrophage-like cells
10   during the exponential and stationary growth phases of *S.
typhimurium* and are consistent with what has been
reported for SL1344-derived *S. typhimurium* containing an
altered *rpoS* allele from *S. typhimurium* LT-2 (Wilmes-
Riesenberg et al., *Infec. Immun.* 65:203-210, 1997).
15   *S. typhimurium* bacteria having an *rpoS* mutation were
also able to survive when internalized in J774 murine
macrophage-like cells or rat bone-marrow derived
macrophages. Rat bone-marrow derived macrophages were
obtained from the femurs and tibias of Sprague Dawley
20   rats (Harlan Sprague Dawley, Indianapolis, IN) and grown
in a 75 cm^2 flask containing Dulbecco Minimal Essential
Medium (DMEM; GibcoBRL, Grand Island, NY) containing 10%
fetal calf serum (FCS), 100 units penicillin/ml and 100µg
streptomycin/ml for 10 days. The macrophages were then
25   cultured in DMEM containing 10% fetal calf serum (FCS),
5% horse serum (HS; Sigma, St. Louis, MO), 10% L-cell-
conditioned medium, 1 mM glutamine, and 1% penicillin for
twenty four hours at 37°C in an environment containing 5%
CO_2. Nonadherent cells were removed, spent medium was
30   replaced, and the cells were incubated an additional five
days. Macrophages were gently scraped from the surface
of the flask, resuspended in fresh DMEM supplemented with

10% FCS, 5% HS and 10% L-cell conditioned medium without antibiotics and used to seed wells of a 24-well microtiter plate prior to infection experiments, at a concentration of 5 X 10⁵ or 1 X 10⁶ cells per ml of rat

5 bone marrow-derived macrophages or J774 cells, respectively.

χ3339 or χ4973 were grown to stationary phase as described above and used in an intracellular survival assay in J774 cells or rat bone-marrow derived

10 macrophages according to Buchmeier et al., *Infect. Immun.* 57:1-7, 1989, with minor modifications. Bacteria were opsonized with 10% normal mouse serum for thirty minutes prior to infection of the monolayers prepared above at a multiplicity of infection (MOI) of between two and ten

15 bacteria per cell. Infected monolayers were incubated for twenty minutes to allow for invasion or phagocytosis, and then washed two times with PBS to remove bacteria remaining in solution phase. Fresh growth media containing 10μg/ml gentamicin was added to washed,

20 infected monolayers to eliminate extracellular bacterial growth. Infected monolayers were incubated for the indicated times after gentamicin addition, washed to remove traces of antibiotic, and then lysed with 0.1% sodium deoxycholate in PBS. Dilutions of lysates were

25 plated onto L agar and incubated at 37°C for twenty four to thirty six hours in order to enumerate surviving intracellular bacteria.

Figures 1 and 2 illustrate the log of the mean and standard deviations of counts of bacteria associated with

30 cells obtained from three wells over the time course of 24 hours. Both χ3339 and χ4973 exhibited a decrease in bacterial cell count during the first two to four hours,

followed by an increase in cell count during the next 20 hours in J774 cells. However, a decrease in the viable number of these bacteria was observed between 4 and 20 hours in rat bone marrow macrophages, yet significant

5   numbers of bacteria survived during the course of the study with little difference between the *rpoS* wild-type or *rpoS* mutant strains. Thus, *S. typhimurium rpoS* mutants are able to survive in either murine macrophage-like J774 cells or in rat bone marrow-derived macrophages

10   as well as their wild-type parent, indicating that the *rpoS* gene product plays little or no role in the survival of the microbe in these macrophages.

Tissue Distribution of *rpoS* mutants after P.O. Infection:

15   To compare the GALT colonization abilities of the *rpoS::RR10* mutant and wild-type strains, animal infectivity studies were performed.

         Bacteria used in these studies were grown aerobically in a volume of 100 ml L broth at 37°C to an

20   optical density of 0.8 as measured at 600 nm. Bacteria were harvested by centrifugation for ten minutes at 7,000 rpm. The cell pellet was resuspended in 1 ml buffered saline with gelatin (BSG).

         Eight- to ten-week old female BALB/c mice purchased

25   from Charles River Laboratories (Wilmington, MA) were either coinfected with both the χ4973 *rpoS* mutant and χ3339 wild-type strains or individually infected with each strain. In each of the coinfection and individual infection experiments, four groups of three mice each

30   were perorally inoculated with approximately equal numbers of bacteria. Mice were euthanized by CO_2 asphyxiation at one hour and at one, three and five days

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after oral inoculation. Organs and tissues of interest
were aseptically removed and homogenized with a tissue
homogenizer (Brinkman Instruments). Five to ten lymphoid
follicles representing the Peyer's patches were collected
5   from each mouse and combined before homogenization.
Homogenates were diluted into BSG and plated onto
MacConkey/1% lactose agar with and without ampicillin.
This allows a comparison between the total number of both
wild-type and *rpoS* mutant *Salmonella typhimurium* which
10   successfully colonize the tissues, to the total number of
rpoS mutant bacteria which successfully colonize the same
tissues. The data for the coinfection and individual
infection experiments are shown below in Tables 5 and 6,
respectively.

Table 5. Ratios of *S. typhimurium* wild-type to *rpoS* mutants in mouse tissues after peroral coinfection[a]

Time after infection	Intestinal Contents	Intestinal Wall[b]	Peyer's Patches	Spleen	Liver
1 hour	1.7 ± 0.4	1.7 ± 0.5	N.D.[c]	N.D.[c]	N.D.[c]
1 day	2.1 ± 0.4	1.5 ± 0.1	1.1 ± 0.10	N.D.[c]	N.D.[c]
3 days	2.1 ± 0.1	8.7 ± 4.3	10.9 ± 5.4	815 ± 743	122 ± 38
5 days	2.7 ± 0.2	6.0 ± 4.2	469 ± 325	250,000 ± 249,800	11,750 ± 10,250

a   Approximately equal numbers of χ3339 (wild-type) and χ4973 *rpoS* (4.5×10^9 and 4.0×10^9, respectively) were administered perorally to 10-week old BALB/c mice. Mean ratios of CFU/g of tissue for χ3339/χ4973 ± SEM (n=3) are given. Only bacterial counts greater than 20 CFU/g were considered when calculating the ratios.

b   Small and large intestine with Peyer's patches removed.

c   N.D., bacterial numbers were not determined.

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Table 6. Colonization of mouse tissues after individual infection with *S. typhimurium* wild-type or rpoS mutant strains[a]

Time[b]	Tissue	Bacterial numbers χ3339	(cfu/g tissue) χ4973
Day 3	Wall[c]	$2.1 \times 10^3 \pm 1.2 \times 10^3$	$2.7 \times 10^3 \pm 6.4 \times 10^2$
	Peyer's patches	$1.7 \times 10^5 \pm 4.1 \times 10^4$	$5.8 \times 10^4 \pm 1.1 \times 10^4$
Day 5	Wall[c]	$1.9 \times 10^4 \pm 6.6 \times 10^3$	$6.5 \times 10^3 \pm 2.5 \times 10^3$
	Peyer's patches	$9.9 \times 10^5 \pm 2.4 \times 10^5$	$4.5 \times 10^4 \pm 1.6 \times 10^4$

a   Ten-week old BALB/c mice were administered perorally with either wild-type χ3339 (2.7×10^9 CFU) or rpoS mutant χ4973 (1.1×10^9 CFU) bacteria. Only bacterial counts greater than 20 CFU/g were considered significant.

b   The intestinal wall and the Peyer's patches were excised after the indicated time. Three mice were euthanized at each time point. Standard errors are shown for each experiment.

c   Small and large intestine with Peyer's patches removed.

The rpoS mutant strain χ4973 and the wild-type strain χ3339 initially colonized the gastrointestinal tract with similar efficiency as judged by the numbers of bacteria associated with the intestinal wall at day three in both mixed (Table 5) and individual (Table 6) infections. Thus the rpoS mutants survived passage through the stomach as well as the wild-type parent strain.

However, the rpoS mutant strain χ4973 was much less efficient in colonizing the Peyer's patches as compared

to its wild-type parent strain, χ3339 (Tables 5 and 6).
This disadvantage of the *rpoS* strain was even more
pronounced in the spleen (Table 5).   Thus, the *S. typhimurium* strain with the *rpoS* mutant allele is
5   defective in its ability to colonize the GALT and the
spleen, which are two primary lymphoid organs in which
immune responses are elicited.

To determine whether the *rpoS* gene product regulates
expression of chromosomally-encoded genes whose products
10 are important for *S. typhimurium* colonization of Peyer's
patches, the wild-type χ3339 and *rpoS* mutant χ4973
strains were cured of their virulence plasmids to
generate plasmid-cured isogenic derivatives χ3340 and
χ8125, respectively.   The ability of these derivative
15 strains to colonize Peyer's patches was examined
following peroral administration of χ3340 and χ8125 in a
1:1 ratio and the data are shown in Table 7 below.

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Table 7. Ratios of wild-type to *rpoS* mutants for
virulence plasmid-cured *S. typhimurium* in mouse tissues
after peroral coinfection[a]

Time after Infection	Intestinal Contents	Intestinal Wall[b]	Peyer's Patches
3 days	37.7 ± 11.8	4.4 ± 3.5	UD[c]
5 days	3.2 ± 1.2	1.2 ± 0.3	5.4 ± 0.5

a   Approximately equal numbers of χ3340 and χ8125 (4.0
        x 10^9 CFU and 3.4 x 10^9 CFU, respectively) were
        administered perorally to 10-week old BALB/c mice.
        Mean ratios of CFU/g of tissue for χ3344/χ8125 ± SEM
        (n=3) are given. Only bacterial counts greater than
        20 CFU/g were considered when calculating the
        ratios.

b   Small and large intestine with Peyer's patches
        removed.

c   Bacterial numbers undetectable at a 1:100 dilution.

        As shown in Table 7, χ8125, the virulence plasmid-
cured derivative of the *rpoS* mutant strain χ4973,
exhibited a reduced ability (ca. 5.1 fold) to colonize
Peyer's patches at 5 days postinfection as compared to
the colonizing ability of χ3340, the virulence plasmid-
cured derivative of the wild-type χ3339 strain. These
data indicate that RpoS regulates expression of
chromosomally-encoded gene(s) whose products are
important for successful colonization of murine Peyer's
patches after oral inoculation.

Effect of RpoS⁻ Strain on Histology of Peyer's Patches:
        Peyer's patches were removed from the intestinal
wall of mice at various times after peroral inoculation
with χ3339 or χ4973 and were immediately fixed in an ice-

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cold solution of 1.5% glutaraldehyde and 1.5%
paraformaldehyde in a 0.1M sodium phosphate buffer, pH
7.4, followed by fixation in 2.5% glutaraldehyde also in
sodium phosphate buffer, pH 7.4 for one hour at room
5 temperature. Thick sliced sections of fixed tissue were
stained with Epoxy Tissue Stain (Electron Microscopy
Sciences, Fort Washington, PA) to locate domes of the
Peyer's patches. Thin sliced sections were examined with
a Hitachi H-600 transmission electron microscope (TEM)
10 operated at 75 kV accelerating voltage.

Observation of sections using light or TEM
microscopy revealed major morphological changes in the
integrity of the Peyer's patch epithelium as early as one
day after oral inoculation with the wild-type virulent
15 strain χ3339 (Figs. 3E and 3F). The destruction of the
follicle-associated epithelium (FAE) at three and five
days after oral inoculation with χ3339 was even more
apparent as seen in Figs. 3G and 3H. The enterocytes had
been completely sloughed from the dome epithelium and
20 extensive tissue necrosis was observed. In addition,
there was a dramatic decrease in cell density of the
Peyer's patch lymphoid follicle tissue five days after
oral inoculation of mice with χ3339 (Figures 3h and 4c).

In contrast, Peyer's patches from mice that were
25 uninfected or infected with the *rpoS* mutant strain χ4973
did not exhibit the dramatic changes in tissue morphology
caused by χ3339 infection. Instead, the integrity of the
dome epithelium was uncompromised and very little
decrease in cell density of the underlying lymphoid
30 tissue was observed at one, three and five days after
oral inoculation (Figs. 3B-3D).

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TEM analysis of Peyer's patch tissue before and five
days after oral inoculation with the *rpoS* mutant χ4973
showed that the FAE remained intact (Figs. 5A and 5B),
whereas the FAE was totally destroyed in χ3339 infected
5   Peyer's patches as early as one day after oral
inoculation (Fig. 5F).

Dramatic morphological changes in the underlying
lymphoid tissue were also clearly apparent when viewed by
TEM. Five days after infection with χ4973, lymphoid
10   cells within the Peyer's patch follicle appeared healthy
and similar in morphology to Peyer's patches from
uninfected mice (Figs. 5A and 5B). In contrast,
extensive changes in gross morphology were observed in
Peyer's patch lymphoid cells five days after infection
15   with χ3339 (Fig. 5C).

These data show that *rpoS* mutant *S. typhimurium* do
not efficiently invade and colonize the GALT. As a
result, the *rpoS* mutants would be expected to be
defective in stimulating a generalized mucosal immune
20   response, which is dependent upon colonization of the
GALT. Furthermore, because the GALT is the portal of
entry into mesenteric lymph nodes and the spleen, the
mutants would also be expected to be ineffective in
invading and colonizing these deeper lymphoid tissues.
25   This would be expected to result in the mutants being
defective in stimulating systemic, humoral immunity as
well as cellular immune responses, which are dependent
upon colonization of the mesenteric lymph nodes and
spleen. In contrast strains containing the wild-type
30   *rpoS* allele more efficiently invade and colonize the GALT
and deeper lymphoid tissues and are, as a result, more

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effective in eliciting mucosal, humoral and cellular
immune responses.

On the other hand, the *rpoS⁺* strains destroyed the
Peyer's patch tissue, making such strains less than ideal

5   candidates for use in vaccines. Therefore, it is
desirable to modify the *rpoS⁺* microbes with at least one
virulence reducing mutation so that the microbes are
still able to invade and colonize the Peyer's patches,
but without destroying the Peyer's patch tissue.

10

Example 2

This example illustrates methods which can be used
in constructing defined deletion mutations in genes to
confer an attuation upon *rpoS⁺ S. typhimurium* and *S. typhi*

15 strains as well as other bacteria suitable for use as
vaccines for humans.

The generation of chromosomal deletions using
transposon Tn*10* has been previously described in a wide
variety of bacteria, including *Salmonella* (Kleckner et

20 al., *J. Mol. Biol. 116*:125-159, 1977; EPO Pub. No.
315,682; U.S. Patent No. 5,387,744; which are
incorporated by reference). Recently, new methods have
become available for introducing specific mutations into
genes. The gene to be mutated can be selected from a

25 population of clones contained in a genomic DNA library
constructed in a cloning vector, or by cloning the
amplified product containing all or a portion of the gene
into a plasmid using PCR methodology. Mutations
introduced into such genes or portions of genes are known

30 as defined deletions and these are constructed using one
of two general methods.

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One method employs restriction enzymes to remove all or a portion of an isolated gene from a recombinant vector. This method allows the mutation of genes for which DNA sequence information is unavailable. However,

5   this method is limited to the use of restriction sites present within the gene or within the DNA flanking the cloned gene.

Another method employs the use of divergent PCR primers synthesized based upon known DNA sequence either

10   within the gene to be deleted or within DNA flanking the gene. The primers are mixed with a vector containing a cloned gene and subjected to an inverse PCR reaction, resulting in the amplification of the entire plasmid but deleting all or a portion of the target gene (Innis et

15   al., *infra*). The PCR reaction amplifies upstream and downstream regions flanking a specified segment of DNA to be deleted from the cloned gene and generates a product. consisting of the cloning vector and upstream and downstream flanking sequences. The inverse PCR method is

20   preferred because it allows the placement of mutations of any size at any position within a gene of known DNA sequence, and allows the introduction of novel restriction sites to be engineered into the PCR primers or target DNA which then can be used for the subsequent

25   insertion of other cloned sequences. An alternative PCR method for generating defined deletions relies on amplified PCR products which represent portions of the gene or flanking DNA sequence. These are ligated together in a cloning vector to construct the defined

30   deletion mutation.

A genomic library can be constructed in any number of cloning vectors (Sambrook et al., supra). Clones

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containing a gene in which a deletion is to be generated
can be isolated from the genomic library by
complementation in a bacterial strain which contains a
mutation in the same gene.

5   For example, genomic DNA libraries from wild-type
Salmonella typhimurium UK-1 (χ3761) can be constructed in
a suitable cloning vector such as pNEB-193 (New England
Biolabs), which is a pUC19 derivative that carries single
sites for the unique 8-base cutters: AscI, PacI and PmeI.
10   Generally, genomic DNA is isolated according to standard
methods (Sambrook et al., Molecular Cloning/A Laboratory
Manual Second Edition, Cold Spring Harbor Press, Cold
Spring Harbor, New York, 1989). Sau3A1 partially
digested genomic DNA is sized on an agarose gel and
15   extracted using commercially available methods in kit
form obtained from Promega, Quiagen, or Bio101. DNA
fragments between 2 and 6 kb are isolated and ligated
into a plasmid first digested with BamHI or BglII, then
dephosphorylated using alkaline phosphatase according to
20   the manufacturers' instructions. The resulting plasmid
library is then introduced into an appropriate E. coli
strain in order to amplify the genomic library and to
obtain a population of recombinant plasmids containing
random genomic DNA inserts ranging in size from 2 to 6
25   kb. Relevant clones are isolated from a genomic library
by complementation of mutant E. coli or S. typhimurium
strains.

       Where the DNA sequence of a gene is already known,
PCR primers are synthesized and the gene and often some
30   flanking sequence is amplified using PCR methodology
directly from a sample of bacteria or from purified
genomic DNA, and the product, cloned into a plasmid

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vector such as pNEB-193. Thus, where the gene sequence
is known, screening a genomic library is not required.

Virtually any cloning vector can be used in
constructing the strains of the present invention, so
5   long as the defined deletion is located on the vector and
is linked to a selectable marker. There are a number of
different methods available for introducing the defined
deletion mutations into the chromosome, including
temperature-sensitive replicons (Hamilton et al., *J.*
10   *Bacteriol. 171*:4617-4622, 1989), linear transformation of
recBC mutants (Jasin et al., *J. Bacteriol. 159*:783-786,
1984), and host restricted replicons known also as
suicide vectors (Miller et al., *J. Bacteriol. 170*:2575-
2583, 1988). All of these methods can result in an
15   allele replacement, whereby a mutant allele constructed
on a vector replaces a wild-type allele on the
chromosome, or vice versa.

The *pir*-dependent R6K replicon has been used by
numerous investigators and is one of the most reliable
20   suicide vectors available for allele replacement.
Replication of the R6K plasmid requires the *pir* gene
product. A *pir*-dependent plasmid will not replicate in a
pir⁻ host bacterium, and so the presence of a defined
deletion mutation on a *pir*-dependent plasmid will allow
25   for the selection of rare events in which the plasmid has
integrated into the host chromosome within a homologous
region flanking the deletion constructed on the plasmid.
This event will confer some selectable phenotype upon the
strain into which the plasmid has integrated, because
30   even though the plasmid cannot replicate, the integration
event provides a mechanism of stable maintenance of the
elements on the plasmid. Antibiotic-resistance elements

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are generally used to select for the presence of the
integrated plasmid, and can be selected from genes which
encode resistance to ampicillin, kanamycin,
chloramphenicol, gentamicin, spectinomycin and

5   tetracycline, and others well known in the art. The host
strain which contains a defined deletion along with an
integrated suicide vector is characterized as a
merodiploid, since it contains two different alleles of
the same gene. Generally, the deletion constructed on

10   the vector will represent a gene deletion and the
integrated product on the chromosome will have the
structure characterized by the presence of a wild-type
allele flanking one end of the integrated vector, and the
defined deletion mutant allele at the other end of the

15   vector. Other constructions are well known in the art.
       Bacteria in which the suicide vector has been
excised from the chromosome along with the antibiotic-
resistance marker can be selected on specialized media.
Two such counter selection methods have been employed to

20   identify these antibiotic-sensitive strains. One method,
which is described in Example 1, relies on fusaric acid
sensitivity of tetracycline-resistant strains. Colonies
which appear on fusaric acid plates are screened for the
loss of tetracycline resistance and the presence of the

25   mutant allele. Another counter selection method takes
advantage of sucrose sensitivity using the *sacRB* system
(Kaniga et al., *Gene 109*:137-141, 1991) in which
expression of levanosucrase in the presence of 5% sucrose
is toxic to cells retaining the *sacB* gene.

30   Following the introduction of any defined deletion
mutant allele into a strain, phenotypes associated with
the mutant gene are characterized using standardized

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tests well known in the art. These tests include
determination of phenotypic reversion frequency,
confirmation of deletion by Southern blot or PCR,
agglutination by O-group specific antisera, production of
5   complete LPS, presence of flagellar H antigen, motility,
plasmid content and confirmation of auxotrophies.

  Mutant strains may be shown by Southern blot to
possess a loss of genetic material corresponding to the
region deleted, as revealed by a mobility shift of DNA
10   relative to the wild-type and the defined deletion mutant
allele constructed on a plasmid. PCR analysis of mutant
strains significantly reduces the time required for
confirming the presence of defined deletions since no DNA
isolation is required and results can be completed in
15   less than one day. The PCR method also allows the
identification of erroneous recombination events or
retention of delivery vector sequences, revealed as
mobility shifts or the production of multiple DNA
fragments other than those expected upon gel analysis of
20   PCR products.

  After construction, strains with defined deletion
mutations are fully evaluated for properties associated
with the mutation and/or which are important for a strain
to be immunogenic as well as attenuated. For example,
25   production of full-length LPS similar to the parental
wild-type strain is evaluated using silver stained gels.
The confirmation of correct O-antigen is determined by
antisera agglutination of mutant cells. Mutant strains
are evaluated for positive agglutination using diluted
30   poly H antiserum (Difco) and subjected to motility tests
in soft agar motility tubes relative to the parent
strains and non-flagellated control strains, χ3420 and

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χ3422. Standard clinical API test strips are used

following isolation of each mutant strain to obtain

fermentation and biochemical data for comparison to

parental strains. Growth rates and plasmid content of

5   the mutant strains are also compared to that of parental

strains. With *S. typhi* strains, the plasmid content is

not evaluated because the large virulence plasmid present

in *S. typhimurium* is absent in *S. typhi* (Gulig et al.,

Infect. Immun. 56:3262-3271, 1987).

10

Construction of Defined Deletions in *phoP*, *phoQ*, and

phoPQ genes

The *Salmonella phoPQ* operon consists of *phoP* and the

adjacent downstream *phoQ* genes. Defined deletions in the

15   *phoP* and *phoQ* genes can be constructed using an inverse

PCR strategy since the entire nucleotide sequence of the

operon and some flanking sequence is known. The DNA

sequence reveals the presence and position of restriction

sites which can be useful in constructing defined

20   deletions in these genes. The genes can be isolated on a

single 2,110 base pair PCR product and cloned into a

plasmid vector. The recombinant vector containing the

phoPQ gene cassette can be digested with restriction

enzymes to delete most of the *phoP* gene, leaving the *phoQ*

25   gene intact. The defined *phoP* deletion on the *phoPQ* gene

cassette can be inserted into a suicide vector, and

introduced into the chromosome of a wild-type *phoPQ*

Salmonella to produce an antibiotic-resistant

merodiploid, which can be grown on appropriate media to

30   select for the loss of the integrated plasmid along with

the antibiotic-resistance marker. Antibiotic-sensitive

strains can be phenotypically characterized for the

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presence of an appropriate defined deletion *phoP* mutant

allele by screening for the loss of acid phosphatase

activity using the agar overlay method of Kier et al. (*J.*

Bacteriol. 130:399-410, 1997).   A mutation in either

5   *phoP* or *phoQ* is sufficient to confer a PhoP⁻ phenotype.

Defined deletion mutants in *phoQ* or in both *phoP* and

phoQ can be generated using a similar strategy, using

restriction enzymes to delete defined segments of DNA

from either *phoQ* or from both *phoP* and *phoQ*, and

10   introduced into the chromosome on a suicide vector to

generate merodiploids, which can be counter selected on

appropriate media for the loss of the integrated plasmid

and antibiotic-resistance marker, and phenotypically

screened for the presence of the relevant defined

15   deletion mutant allele using PCR to verify the genotype.

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Construction of Defined Deletions in the *cya* gene

· A recombinant vector which confers a maltose
positive phenotype to an *E. coli cya* mutant strain when
grown on MacConkey maltose media can be used to construct
5   a defined deletion in a *Salmonella cya* gene. Divergent
primers based on the known *Salmonella cya* gene sequence
can be used in an inverse PCR reaction with the
complementing recombinant vector as a template to
generate a linear product consisting of the vector and
10 DNA flanking either end of the deleted DNA specified by
the PCR primer positions. Alternatively restriction
enzymes can be used to delete all or a portion of the
complementing *cya* gene from the recombinant vector.

A defined deletion constructed using either method
15 can be excised from the cloning vector using restriction
enzymes and introduced into a suicide vector containing
an antibiotic-resistance marker. The resulting
recombinant suicide vector containing the defined
deletion *cya* allele can be introduced into the chromosome
20 of a wild-type *cya Salmonella* strain to generate an
antibiotic-resistant merodiploid. The merodiploid would
be grown on appropriate media to select for the loss of
the integrated plasmid along with the antibiotic-
resistance marker. Antibiotic-sensitive strains would be
25 phenotypically characterized for the presence of an
appropriate defined deletion Δ*cya*-27 mutant allele. MGM-
232 and χ8217 are two *S. typhimurium* UK-1 strains with
defined Δ*cya-27* mutations that were constructed by these
methods (see Table 1).

30 Construction of Defined Deletions in the *crp* gene

Defined deletions in the *Salmonella crp* gene can be
constructed using a strategy similar to that used for

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construction of a defined deletion in *cya*. A recombinant
vector can be selected which confers a maltose positive
phenotype to an *E. coli crp* mutant strain when grown on
MacConkey maltose media. Divergent PCR primers can be
5   used to delete the known *Salmonella crp* gene and flanking
sequences, and the resulting defined deletion introduced
into the chromosome of a wild-type *crp Salmonella* on a
suicide vector to generate an antibiotic-resistant
merodiploid. The merodiploid could be grown on
10   appropriate media to select for the loss of the
antibiotic resistance and the integrated plasmid, and
antibiotic-sensitive strains could be phenotypically
characterized for the presence of an appropriate defined
deletion *crp* mutant allele.

15

Construction of Δ*cya* Δ*crp* double mutants

       Strains which contain defined deletion mutations in
both *cya* and *crp* can also be constructed. For example,
cya mutants can be constructed as described above, and
20   then a defined deletion mutant *crp* allele can be
introduced on a suicide vector to generate merodiploids
selected on an appropriate antibiotic medium. Growth of
merodiploids on an appropriate medium such as fusaric
acid or 5% sucrose as described above can be used to
25   counter select for the loss of the suicide vector along
with the antibiotic-resistance gene from the chromosome,
and an appropriate defined deletion *crp* mutant can be
phenotypically identified on MacConkey maltose medium
containing 2 mM cAMP. This is because cAMP which is the
30   product of adenylate cyclase encoded by the *cya* gene
causes a *cya* mutant to be phenotypically Cya$^+$ on MacConkey
maltose agar. However, a *crp* mutation renders the *cya*

mutant strain no longer capable of fermenting maltose and
thus selection on maltose medium allows easy detection of
strains with both of the defined Δcya and Δcrp mutations.
MGN-431 and χ8214 are two strains with defined cya and
5   crp deletion mutations constructed in this way (see Table
1).

Construction of Defined Deletions in pmi and cdt genes
     Mutations in other genes have also been shown to
10   confer an attenuation on Salmonella, including cdt and
pmi alleles. Defined deletions in the pmi gene can be
constructed using an inverse PCR strategy, or restriction
enzymes. A recombinant vector which confers a mannose-
positive phenotype to an E. coli or Salmonella mutant pmi
15   strain when grown on MacConkey mannose media can be used
to construct a defined deletion mutant pmi allele using
either restriction enzymes or inverse PCR. The defined
deletion pmi allele can be inserted into a suicide vector
and integrated into the chromosome of a pmi⁺ Salmonella to
20   generate an antibiotic-resistant merodiploid, which can
be grown on appropriate media to select for the loss of
the integrated plasmid along with the antibiotic-
resistance gene. Antibiotic-sensitive strains would be
phenotypically characterized for the presence of an
25   appropriate defined deletion pmi mutant allele by
screening for a reversible rough-smooth phenotype,
detecting a smooth phenotype due to the synthesis of LPS
O-antigen repeats when grown in the presence of mannose,
and a rough phenotype when grown in the absence of
30   mannose detected by the absence of agglutination in the
presence of LPS O-antisera.

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A defined deletion which confers a Cdt⁻ phenotype
upon *Salmonella* can be constructed using restriction
enzymes to delete DNA associated with this phenotype from
a recombinant vector which complements a *Salmonella cdt*
5   mutant. The mutant allele constructed using this
strategy can be inserted into a suicide vector and
introduced into the chromosome of a *cdt⁺ Salmonella* to
generate antibiotic-resistant merodiploids. Merodiploids
can be grown on appropriate media to select for the loss
10 of the integrated vector along with the antibiotic-
resistance marker, and antibiotic-sensitive strains can
be phenotypically characterized for the presence of an
appropriate defined deletion *cdt* mutant allele.

15   Example 3
This example illustrates the construction of defined
Δ*phoPQ23* and Δ*asdA16* deletions in *S. typhi* ISP1820 and
Ty2 to produce strains MGN-1191 and MGN-1256,
respectively.
20   The construction of the defined Δ*phoPQ23* Δ*asdA16 S.
typhi* strains in both the ISP1820 and Ty2 backgrounds
involved the use of two suicide plasmids, pMEG-213
containing the Δ*phoPQ23* region and pMEG-006 containing
the Δ*asdA16* region.
25   The Δ*phoPQ23* deletion was obtained by digesting
pMEG-068 with *Eco*RV and *Tth*IIII1 removing the 1103 bp
*Eco*RV-*Tth*III1 fragment encoding the C terminal end of
PhoP and the His region of PhoQ, responsible for
phosphorylation of PhoP (Figure 6). The linearized
30 plasmid was then treated with T4 DNA polymerase and
religated to produce pMEG-210 (Figure 6). The *Bam*HI-*Xba*I
fragment of pMEG-210 containing the Δ*phoPQ23* deletion was

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then inserted in the *pir*-dependent suicide vector pMEG-
149.to produce pMEG-213 (Figure 6). Since pMEG-213 is a
mobilizable suicide vector encoding for the selectable
marker for ampicillin resistance and the counter-
5 selectable marker, levanosucrase, resulting in
sensitivity to sucrose, the plasmid can be conjugated
into any strain desired selecting for ampicillin
resistance followed by counter-selection for the
replacement of the wild-type *phoPQ* genes with the mutant
10 *phoPQ23* in the presence of sucrose. The host strain
responsible for the delivery of pMEG-213 was obtained by
transforming pMEG-213 into the Pir$^+$ Asd$^-$ delivery host
MGN-617 to produce MGN-758.

Introduction of the *ΔphoPQ23* deletion into *S. typhi*
15 ISP1820, χ3744, and *S. typhi* Ty2, χ3769, was accomplished
by conjugating MGN-758 with the wild-type *S. typhi*
parents and selecting for ampicillin-resistant isolates
which grew without DAP (diaminopimelic acid). The
isolates obtained from this conjugation represent the
20 first integration of the *ΔphoPQ23* deletion into the
chromosome producing a duplication of the wild-type gene
with the mutant *ΔphoPQ23*. These isolates, MGN-1037 and
MGN-1017, were then plated on Luria agar with 5% sucrose
to select for loss of the ampicillin-resistant suicide
25 vector. The isolates obtained by this selection were
then screened for acid phosphatase activity using the
agar overlay method of Kier et al. (*J. Bacteriol.*
130:399-410, 1977). The white phosphatase-negative
colonies were then confirmed for the *ΔphoPQ23* phosphatase
30 minus phenotype and stocked as MGN-1038 for *S. typhi*
ISP1820 and MGN-1018 for *S. typhi* Ty2.

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The introduction of a defined *asd* deletion was then
needed to provide a vaccine strain for the delivery of
heterologous antigens. The defined *asd* deletion present
on pMEG-006 was obtained by performing inverse PCR on
5   pMEG-003 to remove the majority of the coding region of
the *asd* gene between 219 and 1460 bp to produce pMEG-006
containing a new unique *Bgl*II site (Figure 7). pMEG-006
has a *pir*-dependent replicon and the tetracycline-
resistance gene from the transposon Tn*10*, but has no
10   mobilization functions to allow conjugation.

Introduction of the defined Δ*asdA16* deletion into
any strain requires plasmid DNA to be electroporated into
the strain desired followed by selection for tetracycline
resistance. The tetracycline-resistant isolates obtained
15   can then be plated on fusaric acid containing media to
select for loss of the tetracycline-resistant elements of
the suicide vector (Maloy et al., *J. Bacteriol. 145*:1110-
1112, 1981) followed by screening for the Asd⁻ DAP-
requiring phenotype. Both MGN-1038 and MGN-1018 were
20   electroporated with pMEG-006 and tetracycline-resistant
isolates obtained. These isolates were then plated on
fusaric acid plates containing 50ug DAP/ml and the
isolated colonies obtained screened for the loss of the
tetracycline-resistance element of the suicide vector and
25   replacement of the wild-type *asd* gene with the Δ*asdA16*
mutation. Isolates were then confirmed for tetracycline
sensitivity and requirement of DAP. An *S. typhi* Δ*phoPQ23*
Δ*asdA16* derivative of each strain was selected for
further work, which are designated herein as MGN-1191
30   (ISP1820) and MGN-1256 (Ty2) (See Table 1).

Example 4

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This example illustrates methods for testing
Salmonella strains for an RpoS⁺ phenotype.

Testing for catalase activity provides one method
for determining the *rpoS* allelic state of strains since
5   RpoS positively regulates expression of hydroperoxidase
II catalase from the *katE* gene in *S. typhimurium*. (Lowen,
supra). Cultures can be analyzed for catalase production
as an indicator of the RpoS phenotype, by adding 100μl of
hydrogen peroxide (H₂O₂) to one milliliter of each strain
10   grown to stationary phase (Mulvey et al., *Gene* 73:337-
345, 1988). Vigorous bubbling of a stationary phase
culture after addition of H₂O₂ would suggest that the
strain contained a wild-type *rpoS* allele, whereas minimal
bubbling would imply that the strain contained a
15   defective *rpoS* allele.

The RpoS phenotype of attenuated *Salmonella* strains
can also be determined by assessing the sensitivity of
the strains to nutrient deprivation and acid and
oxidative stress. *Salmonella rpoS* mutants are known to
20   have a reduced ability to survive these stresses as
compared to wild-type parents (Fang et al., *Proc. Natl.
Acad. Sci. 89*:11978-11982, 1992). Evaluation of
prolonged stationary-phase survival can be performed in
M9 medium on a rotary shaker at 37°C for 6 days.
25   Susceptibility to pH 4.0 can be determined by pelleting
stationary-phase bacteria and resuspending cells in L-
broth adjusted to pH 4.0 with citrate buffer.
Sensitivity of strains to oxidative stress can be
determined by the addition of hydrogen peroxide to
30   stationary-phase bacteria in L-broth to a final
concentration of 15 mM. In each of these experiments,

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bacteria are removed at timed intervals, eluted and
plated onto L-agar for quantitation of viable cells.

The response of an *S. typhimurium rpoS* mutant strain
to each of the above mentioned stresses has been reported

5   and compared to its virulent parent strain (Fang et al.,
supra). Specifically, the *rpoS S. typhimurium* mutant
exhibited a 7-fold reduced ability to survive a prolonged
stationary phase relative to its wild-type parent in M-9
media on a rotary shaker at 37°C for 6 days. Likewise,

10   the *rpoS S. typhimurium* mutant was 10-fold more
susceptible to killing by pH 4.0 as compared to its wild-
type parent after stationary-phase cultures of these
strains were resuspended in L-broth adjusted to pH 4.0
with citrate buffer. In addition, 98% of an inoculum of

15   the *rpoS S. typhimurium* mutant did not survive a 60
minute exposure to 15 mM H_2O_2, whereas the wild-type
parent was unaffected by this treatment.

Another approach that can be used to determine the
RpoS phenotype involves assessing the ability of the

20   *Salmonella* to synthesize glycogen. It has been shown
that *rpoS* null mutations result in a glycogen-negative
phenotype (Lang et al., *Mol Microbiol.* 5:49-59, 1991).
Specifically, the *glgS* gene is an *rpoS*-dependent gene
which is involved in glycogen synthesis. Furthermore,

25   since a null *glgS* mutant accumulates more glycogen than a
rpoS mutant, *rpoS* may have further effects on glycogen
synthesis in addition to *glgS* induction. Thus, it is
possible to determine the *rpoS* allelic state of strains
by analyzing their ability to accumulate glycogen.

30   Accumulation of glycogen is tested by growing cells
as single colonies or patches on Q-3 medium which
contains 0.06M K_2HLPO_4, 0.03M KH_2PO_4, 0.008M $(NH_4)_2SO_4$,

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0.0017M sodium citrate, 8.1 x 10^{-4}M MgSO$_4$, 1.9 x 10^{-4}

histidine HCl, 1.5 x 10^{-5}M thiamine HCl, and 0.056M

glucose. When analyzing the glycogen biosynthesis

abilities of auxotrophic strains, the appropriate

5   nutrient supplements are added to Q-3 media. For

example, methionine (20 µg/ml), threonine (80µg/ml),

leucine (20 µg/ml), and DAP (100 µg/ml) must be added to

Q-3 media to sustain the growth of Δasd mutant strains.

Furthermore, since S. typhi Ty2 and ISP1820 are cys and

10   cys trp mutants, respectively, Q-3 media must be

supplemented with each of these amino acids at a

concentration of 20µg/ml. After about 20 hours of growth

at 37 C, the cells are treated with a solution of iodine

and potassium iodide. Strains which are wild-type and

15   functional with respect to glycogen biosynthesis turn

brown after iodine treatment, while those strains which

are defective in glycogen biosynthesis stain yellow.

In a variation of the above approaches, the RpoS

phenotype can be determined by first moving the rpoS

20   allele into either a wild-type S. typhimurium such as

χ3339 if the allele to be tested is expected to have a

rpoS mutation or into the rpos mutant χ4973 if the allele

is expected to be rpoS$^+$ using P22HTint-mediated

transduction followed by subsequent testing of the

25   derived microbe for RpoS phenotype by any of the tests as

described above. Final proof of the allelic state of an

rpoS allele can be achieved by DNA sequencing using PCR

methods.

30   Example 5

This example illustrates the superior ability of

attenuated S. typhimurium rpoS$^+$ strains having attenuating

mutations in the *cya*, *aroA*, or in both the *cya* and *crp*
genes in colonizing Peyer's patches of the GALT, compared
to the colonizing ability of corresponding *rpoS* mutant *S*.
typhimurium strains.

5   The attenuated *rpoS*[+] *S. typhimurium* strains tested
were MGN-431, a Δ*cya*/Δ*crp* mutant; χ3679, an Δ*aroA* mutant;
and MGN-232, a Δ*cya* mutant (see Table 1 for derivations).
Comparative *S. typhimurium* strains containing an inactive
rpoS allele were constructed as described below to obtain

10   χ8214, χ8215 and χ8217.

Cultures were maintained as frozen cultures
suspended in 1% Bacto-peptone containing 5% glycerol and
fast-frozen in dry-ice ethanol for storage in duplicate
at -70°C and also suspended in 1% Bacto-peptone containing

15   50% glycerol for storage at -20°C for routine use.

Complex medium for routine cultivation of *S.*
typhimurium strains was L broth as described above.
Difco agar was added to Lennox broth at 1.5% for base
agar and 0.65% for soft agar. L agar was used for

20   routine enumeration of bacteria. Fermentation was
evaluated by supplementing MacConkey base agar (Difco,
Detroit, Mich.) with 1% final concentration of lactose.

In generating comparative *rpoS* mutant cultures,
media were supplemented with ampicillin (50μg/ml) to

25   select for ampicillin-resistant *S. typhimurium* strains
containing the inactive *rpoS* allele. Buffered saline
with gelatin (BSG) (Curtiss, 1965 supra) was used
routinely as a diluent.

Bacteriophage P22HT*int* propogated on SF1005 was used

30   to transduce the *rpoS* mutant allele into MGN-431, χ3679,
and MGN-232 to generate χ8214, χ8215, and χ8217,

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respectively (see Davis et al., *supra*). An overnight
culture of the donor strain was diluted 1:20 into
prewarmed L broth, grown for 60 minutes with shaking at
37°C and then infected with P22HT*int* at a multiplicity of

5   0.01. The infection mixture was shaken overnight for
approximately 15 h, chloroform added and allowed to shake
an additional 10 minutes at 37°C, and the suspension
centrifuged (Sorvall RC5C, SS-34 rotor, 7,000 rpm, 10
min) to remove bacterial debris. The supernatant fluid

10 containing the phage (ca. 10^{10}/ml) was stored at 4°C over
chloroform. Ampicillin to a concentration of 50 µg/ml
was used to select for transductants containing an
inactive *rpoS* allele.

The RpoS phenotype of the *S. typhimurium* strains was

15 determined by testing for catalase and glycogen synthesis
activities as described in Example 4. Results are shown
in Table 8.

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Table 8.   Catalase and Glycogen Activity Tests on *S. typhimurium* Strains.

Strain	Relevant Genotype	Catalase Activity	Glycogen Synthesis Activity
χ3339	SL1344 pStSL100$^+$ *hisG rpsL*, colicin$^+$	+	+
χ4973	SL1344 pStSR100$^+$ *hisG rpsL*, *rpoS*::RR10, colicin$^+$	–	–
χ3761	UK-1 wild-type prototroph	+	+
rpoS-	UK-1 UK-1 *rpoS*::RR10	–	–
MGN-232	UK-1 Δ*cya-27*	+	+
χ8217	UK-1 *rpoS*::RR10 Δ*cya-27*	–	–
MGN-431	UK-1 Δ*cya-27* Δ*crp-27*	+	–
χ8214	UK-1 *rpoS*::RR10 Δ*cya-27* Δ*crp-27*	–	–

Those *Salmonella* known to have a wild-type *rpoS* gene showed catalase activity, whereas, those strains having a mutation in the *rpoS* gene showed no catalase activity. Results with glycogen activity testing agreed with catalase testing with the exception that MGN-431, which has an *rpoS* gene and was catalase positive, nevertheless, gave negative results in the glycogen test. This is undoubtedly due to the fact that glycogen synthesis is also dependant on *crp* gene function.

Female BALB/c mice (6 to 10 weeks old) (Charles River Laboratories, Wilmington, Mass.) were used for infectivity and/or immunization experiments. Animals were held for one week in a quarantined room prior to being used in experiments. Experimental mice were placed

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in Nalgene filter-bonnet-covered cages with wire floors.
Food and water were withheld for 4-6 hours prior to
peroral infection.

5   The animal infectivity of *S. typhimurium* strains was
determined following peroral (p.o.) inoculation.
Bacteria for inoculation in mice were grown overnight as
standing cultures at 37°C in L broth. These cultures were
diluted 1:200 into prewarmed broth and aerated at 37°C for
approximately 4 h to an OD_{600} of 0.8. The cells were
10   concentrated 50-fold by centrifugation in a GSA rotor at
7,000 rpm for 10 min at 4°C in a Sorvall RC5C centrifuge
followed by suspension in BSG. Suitable dilutions were
plated on L agar for titer determination. For all p.o.
inoculations with *S. typhimurium*, mice were deprived of
15   food and water for 4-6 h prior to inoculation. They were
then fed 20 µl of *S. typhimurium* suspended in BSG using a
Pipetman P20. Food and water were returned 30 minutes
after oral inoculation.

In order to assess the colonization of the GALT and,
20   in particular, Peyer's patches, by $rpoS^+$ attenuated *S.
typhimurium* strains, three groups of three mice each were
inoculated perorally with equal numbers (approximately 10^9
CFU) of an $rpoS^+$ attenuated *S. typhimurium* strain and its
corresponding *rpoS::RR10* mutant derivative, which were
25   grown according to the conditions described above.
Quantitation of viable *S. typhimurium* in Peyer's patches
was performed as follows. The mice were euthanized at 3,
5, and 7 days after p.o. infection and their Peyer's
patches collected. The Peyer's patches from each mouse
30   were aseptically removed and placed in polypropylene
tubes with BSG, homogenized with a Brinkmann tissue
homogenizer (Brinkmann Instruments) and placed on ice.

Appropriate dilutions of the homogenate were plated on MacConkey agar supplemented with lactose at 1% with and without ampicillin. Differentiation of the strains was facilitated by the presence of an ampicillin-resistance

5 marker within the inactive *rpoS::RR10* allele. Plates were incubated for 12-15 hours at 37°C. Titers in the respective Peyer's patches were determined for each time period and the geometric means calculated for 3 mice per group at each time of sampling.

10 Table 9 below shows the distributions of *rpoS⁺* and *rpoS::RR10* mutant *S. typhimurium* strains containing Δ*cya*/Δ*crp*, Δ*aroA*, or Δ*cya* mutations in murine Peyer's patches after peroral infection.

Table 9

Geometric mean ratios of attenuated $rpos^+$ S. typhimurium Δcya/Δcrp, ΔaroA, or Δcya strains to their corresponding isogenic rpoS::RR10 mutant Δcya/Δcrp, ΔaroA or Δcya derivatives in murine Peyer's patches after peroral coinfection[a]

Time after infection	$rpoS^+$ Δcya/Δcrp / rpoS::RR10 Δcya/Δcrp	$rpoS^+$ ΔaroA / rpoS::RR10 ΔaroA	$rpoS^+$ Δcya / rpoS::RR10 Δcya
3 days	2.1 ± 0.7	1.2 ± 0.2	6.5 ± 2.6
5 days	1,468 ± 1,271	4.3 ± 3.0	1.7 ± 0.8
7 days	308 ± 196	4,135 ± 4,132	7.1 ± 4.2

a Approximately equal numbers of MGN-431 ($rpoS^+$ Δcya/Δcrp) and χ8214 ($rpoS$::RR10 Δcya/Δcrp) (5.2 x 10^8 and 5.4 x 10^8, respectively); χ3679 ($rpoS^+$ ΔaroA) and χ8215 ($rpoS$::RR10 ΔaroA) (6.0 x 10^8 and 6.0 x 10^8, respectively); or MGN-232s ($rpoS^+$ Δcya) and χ8217 ($rpoS$::RR10 Δcya) (6.8 x 10^8 and 6.4 x 10^8, respectively), were administered p.o. to 8-week-old BALB/c mice. Geometric mean ratios ± S.E.M. are given (n=3).

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The *rpoS⁺ S. typhimurium* strain containing Δcya/Δcrp
mutations, MGN-431, exhibited a significantly greater
ability to colonize Peyer's patches at 5 days after oral
infection compared to its *rpoS*::RR10 derivative strain,

5   χ8214. At 3 and 5 days after oral infection, the *rpoS⁺*
ΔaroA *S. typhimurium* strain χ3679 and its *rpoS*::RR10
derivative, χ8215, did not exhibit any significant
differences in ability to colonize the Peyer's patches.
However, by 7 days postinfection, the *rpoS*::RR10 ΔaroA

10   mutant displayed a significantly lower ability to
colonize Peyer's patches as compared to its ΔaroA parent
strain, χ3679.

Coynault et al. have also reported that *rpoS* ΔaroA
derivatives are defective in colonizing murine Peyer's

15   patches compared to *rpoS⁺* parent strains, however, the
decrease in colonization was observed at the earlier
times of 2 and 5 days after oral infection compared to
the decrease in colonization at 7 days reported here.
(Coynault et al., *Mol. Microbiol. 22*:149-160, 1996).

20   Similar studies were done with Δcya mutants. As
shown in Table 9, when administered orally to mice in
approximately a 1:1 ratio, the *rpoS*::RR10 Δcya mutant
strain χ8217 exhibited a reduced ability to colonize
Peyer's patches at 3 and 7 days (ca. 6 and 7 fold,

25   respectively) as compared to its parent strain, MGN-232.

Example 6
This example illustrates the superior balance of
high immunogenicity and low virulence of the *rpoS⁺ S.*

30   *typhimurium* strains of Example 5 having either *aroA* or

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cya mutations, compared to that of the corresponding
isogenic *rpoS⁻* mutant *S. typhimurium* strains.

Protective immunity elicited by attenuated *S.
typhimurium* strains having an *rpoS⁺* genotype compared to
5   the corresponding *rpoS* mutant strains was determined in
BALB/c mice following peroral inoculation as follows.
Five mice per group were p.o. inoculated with 10^6, 10^7,
10^8 and 10^9 CFU of the attenuated *S. typhimurium rpoS⁺*
strain or its isogenic *rpoS* mutant derivative,
10   respectively. Four weeks after immunization, mice were
challenged p.o. with 10^9 CFU of the wild-type SR-11 or
UK-1 virulent parent strain. The degree of protection is
determined by the number of mice alive 30 days after
challenge and the data are shown in Tables 10 and 11
15   below.

Table 10

Protection in mice against challenge with the virulent
wild-type SR-11 strain (χ3181) after immunization with
(A)χ3679 (*rpoS*$^{+}$ Δ*aroA*) or (B) its isogenic *rpoS* mutant
derivative, χ8215

A.　χ3679

Immunization Dose[a]	Challenge Dose of SR-11[a]	Live/Total (%)
1.6×10^6	1×10^9	2/5 (40%)
1.6×10^7	1×10^9	2/5 (40%)
1.6×10^8	1×10^9	4/5 (80%)
1.6×10^9	1×10^9	4/5 (80%)

B.　χ8215

Immunization Dose[a]	Challenge Dose of SR-11[a]	Live/Total (%)
1.4×10^6	1×10^9	0/5 (0%)
1.4×10^7	1×10^9	1/5 (20%)
1.4×10^8	1×10^9	3/5 (60%)
1.4×10^9	1×10^9	3/5 (60%)

a　　Data represented as colony forming units per ml.

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Table 11

Protection in mice against challenge with the virulent
wild-type UK-1 strain (χ3761) after immunization with (A)
MGN-232 (*rpoS*[+] *Δcya*) or (B) its isogenic *rpoS* derivative,
χ8217

A.   MGN-232

Immunization Dose[a]	Challenge Dose UK-1[a]	Live/Total (%)
2.0×10^6	8×10^8	4/5 (80%)
2.0×10^7	8×10^8	5/5 (100%)
2.0×10^8	8×10^8	5/5 (100%)
2.0×10^9	8×10^8	5/5 (100%)

B. χ8217

Immunization Dose[a]	Challenge Dose of UK-1[a]	Live/Total (%)
1.2×10^6	8×10^8	2/5 (40%)
1.2×10^7	8×10^8	1/5 (20%)
1.2×10^8	8×10^8	2/5 (40%)
1.2×10^9	8×10^8	2/5 (40%)

a   Data represented as colony forming units per ml.

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The data presented in Table 10 indicate that, regardless of the dose used for vaccination, mice orally immunized with the $rpoS^+$ $\Delta aroA$ mutant, χ3679 were better
5   protected against oral wild-type challenge than were mice immunized with the isogenic $rpoS$ mutant strain, χ8215. Similarly, Table 11 shows that immunization with $rpoS^+$ microbes attenuated with a Δcya mutation provided better protection against the wild-type challenge than
10   immunization with the isogenic $rpoS$ mutant derivative.

Thus, this study shows that a *S. typhimurium* strain having a functional $rpoS$ gene provides protective immunity that is significantly better than that of the isogenic $rpoS$ mutant strain when challenged orally with
15   the wild-type virulent *Salmonella* strain. Thus, the presence of a functional $rpoS$ allele in *S. typhimurium* increases the immunogenicity of the strain to facilitate the stimulation of a high level of protective immunity.

20   Example 7

This example illustrates the superior immunogenicity of an attenuated $RpoS^+$ strain of *S. typhimurium* following intranasal administration compared to the immunogenicity of the corresponding $RpoS^-$ strain administered by the same
25   route.

Bacteria for intranasal immunization in mice were grown overnight as standing cultures at 37°C in L broth. The following morning, these cultures were diluted 1:200 into L broth and aerated at 37°C until reaching an OD_{500} of
30   0.8. The cells were concentrated by centrifugation in a Sorvall GSA rotor at 7,000 rpm for 10 min at 4°C followed

by suspension in BSG.   Suitable dilutions were plated on
L agar for titer determinations.

Eight-week-old female BALB/c mice were deprived of
food and water for approximately 5 h prior to intranasal

5   immunization.   For each attenuated bacterial vaccine
strain, intranasal immunizations were performed such that
each mouse received either 10^9 or 10^8 cfu in a total
volume of 0.01 ml (10 µl) of BSG using a micropipette.
Immunization was accomplished by inoculating each nostril

10   with 0.005 ml (5 µl) of suspension, or in the case of the
controls with BSG lacking any bacteria.   Food and water
were returned within 30 min following intranasal
immunization.

Intranasally immunized mice and non-immunized

15   controls were orally challenged with either 10^8 or 10^9 cfu
of the wild-type virulent S. typhimurium strain, χ3339,
30 days after the date of intranasal immunization.   The
χ3339 challenge strain was grown overnight as a standing
culture at 37°C in L broth.   The following morning the

20   culture was diluted 1:200 into L broth and aerated at 37°C
until reaching an OD_{600} of 0.8.   The cells were
concentrated by centrifugation in a Sorvall GSA rotor at
7,000 rpm for 10 min at 4°C followed by suspension in BSG.
The mice to be intranasally immunized were deprived of

25   food and water for approximately 5 h prior to the oral
challenge.   Mice were observed over a period of 30 days
for morbidity and mortality.   The data from this
experiment are reported in Table 12.

Table 12

Effectiveness of intranasal immunization with *S. typhimurium* SL 1344 Δcya Δcrp RpoS⁺ vs. Δcya Δcrp RpoS⁻ mutants in protecting female BALB/c mice against peroral challenge with wild-type strain χ3339[a]

Strain	Genotype	Immunizing dose (CFU)	Challenge dose (CFU)	Survivors/ total
χ8296	Δcrp-28	1.2×10^9	1.1×10^9	1/4
	Δcrp-27		1.1×10^8	3/4
	ΔasdA16	1.2×10^8	1.1×10^9	0/4
	RpoS⁺		1.1×10^8	1/4
				5/16
χ8309	Δcrp-28	1.5×10^9	1.1×10^9	0/4
	Δcrp-27		1.1×10^8	1/4
	ΔasdA16	1.5×10^8	1.1×10^9	1/4
	rpoS RpoS-		1.1×10^8	0/4
				2/16
None		BSG	1.1×10^8	0/4

a. Strains were grown in Luria broth with DAP. Preparation of bacterial inocula and animal infection were done as described in text. Oral challenge with *S. typhimurium* χ3339 was given thirty days after intra nasal immunization. Mortality was monitored for thirty days after challenge.

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As shown in the table, intranasal administration of both the RpoS[+] microbe (χ8296) and the RpoS[-] microbe (χ8308) provided some protection against challenge by the wild-type strain (χ3339). The RpoS[+] strain was more

5 effective, however, in that this strain provided greater protection against challenge with the wild-type strain (5 out of 16 survivors) than did the corresponding RpoS[-] strain (2 out of 16 survivors).

The experiment in this example utilized Δcya Δcrp

10 Δasd strains of *S. typhimurium* that were either RpoS[+] (χ8296) or RpoS[-] (χ8309). These microbes did not contain an Asd[+] plasmid vector which would functionally replace the chromosomal Δasd mutation so that they would be expected to death due to inability to synthesize

15 diaminopimelic acid, within the first 24 hours after intransal immunization. This would, in turn, be expected to diminish the immunologic response that would have been elicited by the microbes had they been endowed with an Asd-containing plasmid that would normally be

20 incorporated into a vaccine microbe. Nevertheless, as noted above, 5 of 16 mice immunized with the RpoS[+] strain, χ8296, survived challenge whereas only 2 of 16 mice intranasally immunized with the RpoS[-] strain, χ8309, survived oral challenge with the wild type *S. typhimurium*

25 strain, χ3339. Thus, even during the first 24 hours after administration, the RpoS[+] strain showed a superior ability to elicit a protective immune response.

As has been previously reported in the literature, recombinant attenuated *Salmonella* vaccine strains can be

30 administered by various routes to stimulate mucosal and systemic immunity. For example, Srinivasan et al.

(*Vaccines 95*, R.N.Chanock et al., Eds., Cold Spring
Harbor Laboratory Press, Plainview, NY, p 273-280, 1995)
and Hopkins et al. (*Infect Immun. 63*:3279-3286, 1995)
reported that mice can be immunized not only perorally

5   and intragastrically, but also intranasally,
intravaginally and rectally. Nardelli-Haefliger et al.
(*Infect Immun 64*:5219-5224, 1996) demonstrated that human
volunteers could be immunized rectally with a recombinant
attenuated *Salmonella typhi* vaccine strain. More

10 recently, Galan et al. (*Vaccine 15*:700-708, 1998)
demonstrated that recombinant attenuated *S. typhi* Ty2
strains of an RpoS⁻ phenotype are able to elicit immune
responses when intranasally administered to mice. It is
well known that M cells overlie epithelial lymphoid

15 tissues not only in the small intestine (the so-called
Peyer's patches which are part of the GALT) but also in
the rectum, in the CALT, in the BALT and possibly in
other inductive sites leading to mucosal immune responses
(*Mucosal Immunology, 2nd Edition*, Ogra et al., Eds.

20 Academic Press, San Diego, 1999). The examples above
demonstrated that RpoS⁺ *Salmonella* invade and collonize
epithelial dome M cells in Peyer's patches of the GALT
and elicit an immune response following administration by
the oral route. This example shows that an immune

25 response is also elicited upon administration by the
intranasal route. On the basis of these results, it is
logical to infer that RpoS⁺ *Salmonella* are better able to
attach to and invade M cells overlying lymphoid tissues
in the upper respiratory tract as well as to the M cells

30 of the GALT than are *Salmonella* strains that are
defective with respect to expression of the *rpoS*⁺ gene
(i.e., are RpoS⁻ in phenotype). It, therefore, follows

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that immunization of humans with recombinant attenuated
Salmonella vaccines displaying an RpoS⁺ phenotype would be
more efficacious than those displaying a RpoS⁻ phenotype.
Therefore, RpoS⁺ attenuated *Salmonella* would be superior

5   to RpoS⁻ attenuated *Salmonella* for intranasal, oral,
intragastric and rectal immunization. Since
administration of attenuated *Salmonella* expressing
foreign antigens to colonize mucosal lymphoid tissues is
of paramount importance in eliciting mucosal immunity, it

10   follows that such can be accomplished by use of RpoS⁺
attenuated *Salmonella* of any of various serotypes not
only including *S. typhi* but *S. paratyphi A, S. paratyphi
B,* and *S. paratyphi C,* which are also restricted to
humans, but also attenuated derivatives of such other

15   serotypes of *S. enterica* such as Typhimurium,
Enteritidis, Dublin, and Choleraesuis.

Example 8

This example illustrates the superior ability of

20   RpoS⁺ recombinant attenuated *Salmonella* vaccines to induce
mucosal IgA and serum IgG antibodies to an expressed
foreign antigen compared to that of the corresponding
RpoS⁻ *Salmonella*.

For these studies, the *S. typhimurium* strains used

25   were attenuated with Δcya, Δcrp and Δasd mutations and
were of either an RpoS⁺ phenotype (χ8296; Table 1) or an
RpoS⁻ phenotype (χ8309; Table 1). Both of these strains
were genetically engineered to produce the hepatitis B
virus core (HBVc) particles with pre-S1,S2 fusions

30   according to methods reported in the literature (Schodel
et al., *Infect. Immun. 62*:1669-1676, 1994); from pYA3167
(Nardelli-Haefliger et al., *supra*, 1996). The plasmid

specifying the HBVc preS1,S2 fusion was electroporated
into χ8296 and χ8309 and the resulting strains were
evaluated for production of the HBVc particles with the
preS1,S2 epitopes. Figure 8A depicts Coomassie blue

5   stained SDS gels whereas Figure 8B depicts the results of
analysis of gels by Western blot using a monoclonal
antibody 2A42 from Hybridoma-5520 directed at the preS2
epitope. As shown in the figures, both constructs
produced the fusion protein which is readily detectable

10   on the Coomassie blue gels as well as following Western
blot analysis.

To evaluate the relative immunogenicity of the two
strains, groups of female BALB/c mice (eight-weeks old)
were perorally immunized with 10^9 cfu of the pYA3167-

15   transformed vaccine strain derivatives of χ8296 and
χ8309. According to the immunization schedule described
by Schodel et al.(1994, *supra*), mice were immunized
orally with two doses of vaccine given two days apart. .
Strains were grown in L broth as standing overnight

20   cultures at 37°C. In the morning, 1:200 dilutions into L
broth were grown with moderate aeration until achieving
an OD_{600} of 0.8. Bacteria were sedimented by
centrifugation and suspended in BSG to desired densities
so that the vaccine dose could be administered in a

25   volume of 0.02 ml (20 µl). Food and water were withdrawn
from the mice approximately 5 h prior to peroral
immunization and were returned 30 min after immunization.
Serum samples and vaginal washings were collected 4 and 6
weeks after initial immunization (for methodology, see

30   Zhang et al., *Biol. Reprod. 56*:33-41, 1997). Serum IgG
antibody and IgA antibody in vaginal washings were

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detected by ELISA measuring antibody to a full-length
pre-S protein (histidine fusion).

The protocol for ELISA was as follows. Ninety-six-
well Immulon-1 plates (Dynatech, Chantilly, VA) were
5   coated with 10 μg of recombinant HBV pre-S protein
(awd)/ml in 0.2 M bicarbonate/carbonate buffer (pH 9.6)
at 4°C overnight. Nonspecific binding sites were blocked
with 1% BSA in phosphate buffered saline (PBS) + 0.1%
Tween20 (pH 7.4) (blocking buffer) at room temperature
10   for 1 h. Serum samples and vaginal washings were diluted
1:100 and 1:10, respectively, in blocking buffer. One
hundred microliters of the diluted samples were added in
duplicate to the plates and incubated at 37°C for 2 h.
The plates were then washed with PBS + 0.1% Tween20 three
15   times. One hundred microliters of biotin-labelled goat
anti-mouse IgA or IgG were added, respectively, and
incubated at 4°C overnight. Alkaline phosphatase-labelled
ExtrAvidin (Sigma) was added to the plates and incubated
at room temperature for 1h. Substrate solution (0.1 ml)
20   containing p-nitro-phenylphosphate (1 mg/ml) in 0.1 M
diethanolamine buffer (pH 9.8) was added and the optical
density of the resulting substrate reaction read at 405nm
with an automated ELISA reader (BioTech, Burlington, VT).
All the reagents were purchased from Sigma (St. Louis,
25   MO).

The results of the antibody titer determinations are
in Figure 9. As shown in the figure, the RpoS[+]
recombinant attenuated vaccine strain, χ8296, induced
significantly higher antibody titers against the
30   recombinant HBVc preS1, S2 antigen than did the
corresponding RpoS[-] microbe, χ8309, both in serum and in
vaginal secretions at 4 and 6 weeks following peroral

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immunization. It is, therefore, evident that recombinant
attenuated *Salmonella* vaccine strains of an RpoS⁺
phenotype are not only superior in inducing protective
immunity against *Salmonella* as was shown in Example 7

5 above, but they are also superior in inducing immune
responses against expressed foreign antigens.

Example 9

    This example illustrates the method for screening

10 for vaccine strains containing an RpoS⁺ phenotype.
    The evaluation of strains for RpoS⁺ phenotype allows
the identification and selection of RpoS⁺ strains. Such
strains would be expected to show high immunogenicity.
Strains for testing in a screening system for RpoS⁺

15 phenotype can be from any source. For example, strains
obtained from depositories can be tested as illustrated
below.
    Testing for catalase activity and glycogen
biosynthesis was performed as described in Example 4

20 above.
    The results of testing for catalase or glycogen
synthesis activity in typical *S. typhi* strains are shown
below in Table 13. Strains χ8205 and χ8208 did not show
catalase activity which is consistent with earlier

25 reports that these microbes are *rpoS* mutants (Robbe-Saule
et al., *FEMS Microbiol. Lett. 126*:171-176, 1995; Coynault
et al., *Mol Microbiol. 22*:149-160, 1996). The results of
the catalase test suggest that strains χ8204 and χ8207
may also have *rpoS* mutations, however, the glycogen test

30 was positive for these two strains suggesting that the
two strains have an intact *rpoS* gene. A final decision
as to the *rpoS* allelic state in these two strains would,

therefore, require use of other tests as described in
Example 4. The remaining strains for which results were
obtained in both the catalase and the glycogen test
showed corresponding results in both tests.

5

Table 13
Catalase Test on ATCC *S. typhi* strains

Strain	Relevant Genotype	Catalase Activity[a]	Glycogen Synthesis Activity	Source/ Reference
χ3743	ISP1804 Type 46	±	+	Received from D. Hone, Center for Vaccine Development, MD; 1983 isolate from Chilean patient.
χ3745	ISP1822 Type E1	+	+	Received from D. Hone, Center for Vaccine Development, MD; 1983 isolate from Chilean patient.
χ3746	ISP282S Type E1	+	+	Received from D. Hone, Center for Vaccine Development, MD; 1983 isolate from Chilean patient.
χ8203	*cys, trp*	+	+	ATCC 9992V
χ8204	*cys, trp*	–	+	ATCC 33458
χ8205	Ty21a *galE, rpoS, cys, trp*	–	No Growth	ATCC 33459
χ8206	*cys, trp, aroA serC, purA*	+	+	ATCC 39926
χ8207	*cys, trp*	–	+	ATCC 10749
χ8208	Ty2 *cys, rpoS*	–	–	ATCC 19430
χ8209	*cys, trp*	+	+	ATCC 9993
MGN-1256	Ty2 *rpoS cys ΔphoPQ23 ΔasdA16*	–	–	Megan Health, Inc.
MGN-1191	ISP1820 *cys trp ΔphoPQ23 ΔasdA16*	+	+	Megan Health, Inc.

[a] Vigorous bubbling upon addition of H_2O_2 is indicated by +, an intermediate level of bubbling is indicated by ±, and little or no bubbling is indicated by -.

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Example 10

This example illustrates a method that can be used
to introduce a wild-type *rpoS* allele into RpoS⁻ *S. typhi*
strains such as χ3769, MGN-1018 or χ8280 using an allelic
5   replacement strategy.

The wild-type *rpoS* gene can be introduced into the
chromosome of χ3769, MGN-1018 or χ8280 by allelic
exchange using the suicide properties of the R6K-based
plasmid pMEG-149 or its derivative pMEG-375. Plasmids
10 pMEG-149 and pMEG-375 are mobilizable suicide vectors
which carry a λ*pir*-dependent R6K replicon and thus
require a host with the *pir* gene present in *trans* to
allow replication. In addition, pMEG-149 encodes the
selectable marker for Ap^r and the counterselectable
15 marker, levanosucrase whereas pMEG-375 also contains the
cat gene specifying resistance to chloramphenicol (Cm^r).
Since pMEG-149 and pMEG-375 cannot replicate in strains
lacking the *pir* gene, selection of Ap^r and Ap^r Cm^r
transconjugants, respectively, demands the integration
20 of the plasmid into the chromosome, an event which
usually takes place through homology in the inserted
fragment.

Plasmid pSK::*rpoS* contains the entire 1.7 kb *S.
typhimurium* 14028 *rpoS* gene cloned into the *Eco*RV site of
25 pBluescript/SK. The *Eco*RI-*Hind*III fragment containing
the wild-type *rpoS* allele from pSK::*rpoS* was treated with
T4 DNA polymerase and cloned into the *Sma*I site of the
suicide vector pMEG-149. The resulting recombinant
vector carrying the wild-type *rpoS* allele designated as
30 pYA3433 (figure 11), would be introduced into the λPir⁺
Asd⁻ delivery host strain, MGN-617. This strain allows
the conjugal transfer of any plasmid containing an IncP

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mob region to any Asd⁺ recipient, followed by elimination
of the donor on any media lacking diaminopimelic acid
(DAP).

Since duel selection for two drug-resistance genes
5   often enhances selection of merodiploid strains that have
integrated a suicide vector into the chromosome by
eliminating background growth that sometimes occurs when
using Apʳ alone due to the ability of β-lactamase to
rapidly destroy the ampicillin in the selective medium,
10 we also made a suicide vector with the *rpoS⁺* gene using
pMEG-375 which specifies chloramphenicol resistance in
addition to ampicillin resistance. The 1.409 kb *S.*
typhimurium UK-1 *rpoS⁺* gene was recovered from pMEG-328 by
digestion with *Pme*I and *Sma*I and cloned into pMEG-375
15 digested with the same two enzymes. The resulting
suicide vector plasmid, pYA3467, is depicted in Figure
10.

Plasmid pYA3467 carrying the wild-type *rpoS* allele
was introduced via MGN-617 into the Δ*phoPQ* Δ*asd* *S. typhi*
20 Ty2 strain, MGN-1018, by electroporation. Transformants
were selected by spreading on L-agar plates supplemented
with DAP (100μg/ml), ampicillin (50 μg/ml) and
chlormphenicol (40 μg/ml) followed by incubation
overnight at 37°C. Ampicillin- and chloramphenicol-
25 resistant isolates obtained from this transformation
procedure represent the integration of the entire plasmid
including the wild-type *rpoS* allele into the chromosome
by a single crossover event. Such isolates contain two
copies of the *rpoS* gene, ie. a wild-type and a mutated
30 *rpoS* allele. The isolates were then screened on Luria
agar supplemented with DAP (100 μg/ml) and containing 5%
sucrose to select for loss of the suicide vector

sequences by a second crossover event. Sucrose-resistant
isolates were screened for sensitivity to ampicillin and
chloramphenicol and for the presence of a functional *rpoS*
allele (using the catalase or glycogen synthesis test).

5   After identifying bonifide *rpoS*[+] derivatives,
complete characterization is done to verify the presence
of LPS, Vi antigen, all attenuating mutations and the
presence of all other traits that are characteristic of
an RpoS[+] derivative of the MGN-1018 parent. One such

10   derivative was selected as χ8434 (Table 1). Since the
wild-type *S. typhi*, Ty2 strain may possess excellent
attributes as a recombinant attenuated *Salmonella* vaccine
vector if provided with an RpoS[+] phenotype, the wild-type
Ty2 strain χ3769 was also endowed with the *rpoS*[+] gene from

15   pYA3467 using the method described above to generate the
RpoS[+] derivative χ8438 (Table 1). This strain can now be
attenuated by introducing various defined deletion
mutations as described in Examples 2 and 3 and then
endowed with the ability to express various antigens as

20   described in Example 11 below.

          In order to further validate the method, pYA3467
was transferred by the donor MGN-617 to the candidate
recombinant attenuated vaccine strain χ8280 [Ty2 Δ*phoPQ23*
rpoS Δ*asdA16* (pYA3167)]. This strain synthesizes the

25   hepatitis B virus core with pre S1, S2 epitopes due to
the presence of pYA3167. Using the procedures described
above, an RpoS[+] derivative was isolated and fully
characterized. This was designated χ8435 (Table 1).

          The abilities of these three RpoS[+] strains,

30   constructed by introducing a recombinant wild-type *rpoS*[+]
gene to replace the defective *rpoS* mutant gene present in
S. typhi Ty2 and its descendents, to synthesize glycogen

and to give a positive catalase test are shown in Table
14.

Table 14

Test for RpoS Phenotype in Recombinant *Salmonella* Strains
and Their Parents.

Salmonella typhi Ty2	Glycogen Accumulation/ Biosynthesis	Catalase Activity
χ3769 *S. typhi* Ty2 wild-type	−	−
χ8438 *S. typhi* Ty2 *rpoS*+	+	+
MGN-1018; *S. typhi* Ty2 Δ*phoPQ23*	−	−
χ8434 *S. typhi* Ty2 Δ*phoPQ23* *rpoS*+	+	+
χ8280(pYA3167) *S. typhi* Ty2 Δ*phoPQ23* Δ*asdA16*	−	−
χ8435(pYA3167) *S. typhi* Ty2 Δ*phoPQ23* Δ*asdA16* *rpoS*+	+	+

This method can also be used to introduce a
recombinant wild-type *rpoS* gene into various Ty2 derived
vaccine strains such as ATCC 55117 (χ3927; Δ*cya-12* Δ*crp-11*) or ATCC 55118 (χ4073; Δ*cya-12* Δ[*crp-cdt*]-10) to
improve the balance between attenuation and
immunogenicity.

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Example 11

This example illustrates the construction of
recombinant attenuated $rpoS^+$ S. typhi strains expressing
foreign antigens for use as oral vaccines to immunize
against various infectious diseases.

The $rpoS^+$ vaccine strains are prepared based upon
S. typhi strains containing a functional rpoS gene such
as ISP1820 using defined deletions as described above in
examples 2 and 3 or based upon attenuated rpoS mutant
strains such as Ty2 which have a recombinant rpoS gene as
described in example 8 above. In the construction of
vaccines expressing foreign antigens, the preferred
approach is to use a balanced, lethal host-vector system
which confers stable maintenance and high-level
expression of cloned genes on recombinant plasmids. For
this, a chromosomal mutation of the asd gene encoding
aspartate ß-semialdehyde dehydrogenase is introduced into
the RpoS$^+$ strain to impose an obligate requirement for
diaminopimelic acid (DAP) which is an essential
constituent of the rigid layer of the bacterial cell wall
and which is not synthesized in humans. The chromosomal
Δasd mutation is then complemented by a plasmid cloning
vector possessing the wild-type asd^+ gene as well as a
recombinant gene encoding the desired foreign antigen.
Loss of the plasmid results in DAP-less death and cell
lysis. Such balanced-lethal host-vector combinations are
stable for several weeks in the immunized animal host and
elicit immune responses against the cloned gene product
as well as against Salmonella.

The construction of a defined deletion in the
chromosomal asd gene is described in example 3 above.
The ISP1820 derivative, MGN-1191 and the Ty2 derivative,

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MGN-1256, which have Δ*phoPQ23* and Δ*asdA16* mutations were thus produced. The *asd*-complementing plasmid containing a recombinant gene encoding the desired foreign antigen can be constructed as described in U.S. Patent No.

5   5,672,345. For example, one such plasmid expressing the Hepatitis B virus antigenic nucleocapsid pre-S1 pre-S2 (HBcAg-pre-S) particles, designated as pYA3167, has been constructed as reported in the literature (Schodel, et al., 1996, in Novel strategies in design and production

10   of vaccines. S. Cohen and A. Shafferman, eds., Plenum Press, New York). Accordingly, *S. typhi* MGN-1191 and MGN-1256 have been transformed with plasmid pYA3167 via electroporation. Immunoblot analysis with HBV pre-S2-specific monoclonal antibody was used to determine the

15   level of expression of the hybrid core pre-S gene in the transformed attenuated *S. typhi* carrier strains derived from MGN-1191 and MGN-1256. The expression of the hybrid HBcAg-pre-S antigen in Δ*phoPQ* Δ*asd* mutant *S. typhi* strains was determined as follows. Proteins from whole

20   bacterial cell lysates after overnight culture were separated using 12% sodium dodecyl sulfate (SDS-12%), polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue. Results are shown in Figure 12. Three transformants of MGN-1191 and three

25   transformants of MGN-1256 were studied all of which showed a band at the position of the recombinant antigen (see arrow in figure 12). The MGN-1191 transformant #1 (lane 3) was designated χ8281 and the MGN-1256 transformant #1 (lane 7) was designated χ8280. Both

30   strains express the Vi capsular antigen as determined by positive agglutination with Vi antiserium (Difco).

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In addition, the expression of the recombinant
antigen was assessed by immunoblotting. For
immunoblotting, cells from overnight cultures were taken
up in 2X sample buffer and boiled for 10 minutes to lyse
5   the cells. Proteins were separated by SDS-12% PAGE. The
proteins were subsequently transferred to nitrocellulose;
incubated with monoclonal antibodies specific for HBV
pre-S2; developed with peroxidase-coupled goat anti-mouse
immunoglobin G (IgG) (heavy and light chains) and
10   visualized on X-ray film (Kodak) after incubation with a
chemiluminescent substrate (ECL; Amersham). Results are
shown in Figure 13. As was seen with Coomassie staining,
the three transformants of MGN-1191 including χ8281 (lane
3) and the three transformants of MGN-1256 including
15   χ8280 (lane 7) all showed a band at the position of the
recombinant antigen (see arrow in Figure 13).

For immunoscreening, the following procedure can
be used. Bacterial colonies are lifted onto
nitrocellulose filters and lysed in 1% SDS for 30 minutes
20   at 70°C. Free binding sites on nitrocellulose are blocked
by 10% horse serum in Tris-HCl-buffered saline.
Subsequently, immunoscreens are treated like immunoblots
and secondary goat anti-mouse IgG (heavy and light
chains) is visualized with nitroblue tetrazolium-5-bromo-
25   4-chloro-3-indolylphosphate toluidinium (Promega).

S. typhi rpoS$^+$ strains expressing foreign antigens
can also be constructed using plasmid vectors with
selectable markers other than Asd$^+$, including genes that
confer resistance to drugs such as ampicillin and
30   tetracycline. In addition, the recombinant vector
encoding the desired foreign antigen may be constructed
using well known techniques such that the vector will

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insert into the bacterial chromosome by homologous
recombination or by transposition.

Example 12

5   This example illustrates methods which can be used
in constructing recombinant attenuated vaccine strains
that express foreign proteins so as to suppress,
modulate, or augment immune responses in a beneficial
way.

10   It is well known that live attenuated bacterial
vaccines induce long-lasting immunity by inducing T
helper lymphocyte memory functions. *S. typhimurium*
infection of mice leads predominantly to a Th-1 type of
response although a Th-2 response with production of SIgA

15 in mucosal secretions and serum antibodies against
Salmonella and against foreign expressed antigens is
also induced. IL-10 can be detected at levels indicating
the occurrence of the Th-2 response (Van Cott et al., *J.
Immunol. 156*:1504-1514, 1996). It also known that the

20 recombinant attenuated *S. typhimurium* vaccine can also
induce a CTL response involving CD-8$^+$ cells against a
foreign antigen (Sadoff et al., *Science 240*:336, 1988).
In many cases, however, it would be desirable if a
recombinant attenuated *Salmonella* vaccine elicits

25 predominantly a Th-2 type of response to enhance mucosal
immunity by the production of SIgA and a cellular memory
response for that SIgA production. The lymphokines IL-4
and IL-5 when produced, potentiate such a Th-2 response.
On the other hand, it is desirable in other instances to

30 maximize the ability of the recombinant attenuated
Salmonella to induce a Th-1 type of response which might
be particularly important in providing protective

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immunity against a facultative or obligate intracellular parasite whose antigens are expressed by the recombinant attenuated *Salmonella* vaccine. Shifting the immune response to a predominantly Th-1 or to a Th-2 type of

5   response can be achieved in part by expressing lymphokines via recombinant attenuated *Salmonella* strains. Thus, we have constructed *Salmonella* strains expressing IL-2 which enhances the Th-1 type of response and also potentiates a CTL response which is important in

10 designing attenuated *Salmonella* vaccines to be protective in combating certain types of cancer (Saltzman et al., *Cancer BioTher. Radiol. Pharm.* 11:145-153, 1996; Saltzman et al., *J. Pediatric Surg.* 32:301-306, 1997). Generating the *Salmonella* to induce a predominant Th-2 response can

15 be achieved by causing the strains to express IL-4 and IL-5 as has been done for the latter lymphokine by Whittle et al. (1997, *J. Med. Microbiol.* 46:1029-1038). IL-4 has been expressed by a recombinant *aroA* attenuated *Salmonella* vaccine strain but was not effective since it

20 was not secreted (Denich et al., *Infect. Immun.* 61:4818-4827, 1993). Methods such as described by Hahn et al. (*FEMS Immunol. Med. Microbiol.* 20:111-119, 1998) are now available to succeed in such secreted expression of lymphokines by attenuated *Salmonella.* It is also

25 possible to coexpress peptides such as factor P which is reported to stimulate the secretion of SIgA. Genes for cDNAs have been obtained which specify many different lymphokines, cytokines and other peptide or protein molecules which act to modulate the immune response. It

30 is anticipated that these peptides or proteins could be coexpressed by recombinant attenuated *Salmonella* vaccine strains expressing some antigen from a particular

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pathogen or from a tumor cell line or some other molecule
that was targeted for an immune response that would
induce an immune response to protect against an
infectious disease or to therapeutically correct against

5   a systemic disease of the immunized human. Thus IL-6 has
been expressed and in some cases secreted by recombinant
attenuated *Salmonella* (Dunstan et al., *Infect Immun*
64:2730-2736, 1996; Hahn et al., *FEMS Immunol Med*
Microbiol 20:111-119, 1998). The genes for murine

10 macrophage inhibitory factor (MIF), IL-2, IFN-γ or TNF-α
were individually cloned and expressed by recombinant
attenuated *Salmonella* to alter immune responses against
Leishmania major infection (Xu et al., *J. Immunol.*
160:1285-1289, 1998). TGF-β has also been expressed in

15 recombinant attenuated *Salmonella* vaccine strains to
decrease the inflammatory response by inhibiting
endogenous synthesis of IL-2 and INF-γ but enhancing
synthesis of IL-10 (Ianaro et al., *Immunology 84*:8-15,
1995). Based on data presented in preceding examples, it

20 is evident that recombinant attenuated *Salmonella*
vaccines of the RpoS$^+$ phenotype will be superior to
vaccine strains of an RpoS$^-$ phenotype in expressing
cytokines and other immunoactive molecules to suppress,
enhance and/or modulate the immune response in a desired

25 way.

                         Example 13
     This Example illustrates the use of attenuated RpoS$^+$
Salmonella strains having high immunogenicity and low
virulence, to express an autoantigen and to exert an

30 antifertility benefit.
     We have previously described how to use recombinant
attenuated *Salmonella* strains to express autoantigens so

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as to induce a state of infertility. This technology is
disclosed in U.S. patent 5,656,488. In addition,
Srinivasan et al. (*Biol. Reproduct. 53*:462-471, 1995)
describes how to express a sperm-specific antigen from a
5   recombinant attenuated *Salmonella* so as to induce
antibodies against that sperm antigen to effectively
block the sperm-egg interaction in the mouse to induce a
state of infertility. The specific antigen was specified
by a murine cDNA sequence and the recombinant *Salmonella*
10   was able to induce in mice an immune response against
that autoantigen. Similarly, Zhang et al. (1997, Biol.
Reproduct 56:33-41) expressed in an attenuated *S.
typhimurium* strain, the murine cDNA sequence encoding the
zona pellucida antigen, ZP-3. Mice immunized with
15   *Salmonella* expressing this autoantigen mounted an immune
response to ZP-3. Antibodies to ZP-3 coated the surface
of ova in the ovary and effectively reduced the ability
of sperm to fertilize such eggs. It is also well known
that fusion of an autoantigen to a carrier antigen which
20   is heterologous to the host can lead to the induction of
an immune response which recognizes the autoantigen as
well as the heterologous carrier. In the case of
fertility, such immunization strategies could lead to the
development of contraceptive vaccines.

25

Example 14

This Example illustrates the use of an attenuated
RpoS$^+$ *Salmonella* vaccine engineered to express an allergen
and to induce an immune response to ameliorate the effect
30   of that allergen.

Allergies to pollens, mold spores, insect parts,
animal dander and the like are due to the inhalation of

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air and/or ingestion of food containing such allergens.
The allergies that result are associated with a presence
of IgE antibodies that bind to allergens which activate
mast cells for release of histamines. As is well known,

5   desensitization against allergens can be achieved by
repetitive parenteral immunization of extracts containing
the allergen. Likewise, it is known that oral ingestion
of raw honey containing pollens can be used to
effectively induce a state of tolerance against those

10   allergens. Oral ingestion with such allergens can on the
one hand induce an SIgA response that could block the
ability of allergens to react with IgE and mast cells or
if administered in sufficient quantity could serve to
suppress the synthesis of IgE antibodies, that is to

15   induce tolerance. Since the specific allergenic molecule
in many allergens has been identified and the cDNA cloned
to obtain the nucleotide sequence specifying the
allergen, it is now possible to genetically engineer
heterologous host cells to express the allergen (see for

20   example, Valenta et al, *Allergy 53*:552-561. 1998; Olsson
et al., *Clin. Exp. Allergy 28*:984-991. 1998; Soldatova et
al., *J. Allergy Clin. Immunol. 101*:691-698, 1998;
Asturias et al, *Clin. Exp. Allergy 27:1307-1313*; Twardosz
et al, *Biochem. Biophys. Res. Comm. 239*:197-204, 1997).

25   Accordingly, the attenuated RpoS$^+$ *Salmonella* of the
present invention can be engineered to express an
allergen, possibly in a modified immunogenic but
nonallergenic form to induce a state of tolerance or to
actively promote the production of SIgA against the

30   allergen. The RpoS$^+$ attenuated *Salmonella* described
herein have been shown to be effective in eliciting
immune responses and, hence, it follows that use of such

RpoS[+] *Salmonella* to express modified allergens would be
likely to be effective in ameliorating the consequences
of exposure of humans to allergens by inhalation or
ingestion.

5

Example 15

This example illustrates a procedure that can be
used for testing the safety, immunogenicity and efficacy
of live oral vaccines comprising recombinant attenuated

10 *rpoS*[+] *S. typhi* carrier strains which express a desired
foreign antigen.

Strains tested are attenuated derivatives of
ISP1820 and ISPI822 or attenuated derivatives of Ty2
strain χ8438 (Table 1) containing a recombinant *rpoS*

15 gene.

The Individuals Studied: The individuals studied
are volunteers who are healthy adult humans age 18-40
years of either sex. The prospective volunteers are
screened before the study. The inclusion criteria

20 includes:

1. general good health;

2. evaluation of medical history;

3. normal and regular bowel habits;

4. normal physical examination;

25   5. normal laboratory findings including:

    normal urinalysis,

    normal complete blood count and differential,

    normal blood chemistries (SGPT, alkaline

        phosphatase, BUN, creatinine, fasting

30   blood glucose),

    negative ELISA for HIV-1

    negative pregnancy test (females);

6. able to understand and comply with required

   procedures including the practice of good

   hygiene, maintenance of daily logs and

   willingness to undergo stool collection.

The exclusion criteria includes:

1. history of gall bladder disease;

2. gastric achlorhydria (frequent antacid, H^2

   blocker or B_{12} usage);

3. history of immunodeficiency;

4. positive pregnancy test (females)

5. medical, psychiatric or occupational condition

   which would preclude compliance with protocol;

6. diarrheal illness;

7. history of antibiotic therapy within 7 days

   prior to immunization;

8. history of drug allergy or serious adverse

   reaction to vaccines.

Volunteers are screened and informed written consent is obtained.

Study Design: Groups of 5 or 6 volunteers are studied for each strain and dose. In the first group of volunteers, the subjects will receive a single dose of 10^5 CFU of the attenuated vaccine. If this group develops no clinical symptoms of disease, an escalation in dose will proceed in subsequent groups to establish the maximal safe and minimal immunogenic dose. Subsequent groups will receive 10^6 CFU or greater doses up to a maximal dose of 10^9 CFU.

Preparation of the vaccine inocula: Stock cultures of the *S. typhi* candidate vaccine strains are stored as a cell suspension in 1% bactopeptone (Difco) containing 5% glycerol at -70°C. To make an inoculum of the strain, the

suspension is thawed and then diluted to the appropriate CFU/ml for the particular dose.

Inoculation of Volunteers: On the day of inoculation of volunteers, blood, urine and stool samples are

5   obtained and baseline values for clinical laboratory parameters are determined. In addition, immunoglobins are measured in serum and stool samples. The subjects receive nothing by mouth for 90 minutes before inoculation. Two grams of $NaHCO_3$ are dissolved in 5

10 ounces of distilled water. The subjects will drink 4 ounces of the bicarbonate water and one minute later the subjects will ingest the vaccine suspended in the remaining one ounce of bicarbonate water. Subjects will take no food or water for 90 minutes after inoculation.

15 Clinical monitoring of volunteers: The volunteers are followed as inpatients for a minimum of two weeks and thereafter as outpatients up to a total of four weeks. During this period observations are made for any adverse effects including but not limited to fever, headache,

20 chills, vomiting, diarrhea and abdominal pain. Blood and stool samples are obtained during the testing period and cultures and antibody determinations are done. In addition, PCR for vaccine strain is done on serum. Any volunteer who develops a temperature of 100.8°F at any

25 time during the study will have stool samples and blood drawn for culture; if the temperature remains elevated for 12 hours and/or blood culture is positive, a 10 day course of oral antibiotics will be given.

Procedures for Specimen Collection.

30 Stool Specimens: A record will be kept of the number, consistency and description of all stools passed by volunteers for 14 days post vaccination. Stool volume

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will be measured and the stool will be graded on a 5
point system:

    Grade 1 - firm stool (normal)

    Grade 2 - soft stool (normal)

5   Grade 3 - thick liquid (abnormal)

    Grade 4 - opaque watery (abnormal)

    Grade 5 - rice water (abnormal)

Stool cultures will be performed on a sample of stool (or
rectal swab if stool was not passed) each day on

10 consecutive days for *Salmonella* until negative times one.

    Phlebotomy: Serum (20 ml blood) will be collected
for prescreening evaluation. Serum for antibody (10 ml
blood) determinations will be obtained on days 0, 7, 14
and 28. Heparinized blood for lymphocyte separation (30

15 ml) for antibody-secreting cell assays by ELISPOT will be
collected on days 0, 7, 14 and 28 on a subset of
volunteers. The subset will consist of 2 volunteers in
groups 3, 4 and 5. Volunteers will be selected randomly
by the computer. Blood (10 ml) will be obtained for

20 culture on each day until negative during the post
immunization observation period to detect viable vaccine
organisms by both conventional culture and PCR. In
total, no more than 450 ml of blood will be collected
from any volunteer during any 2 month period. Bacteria

25 in positive blood cultures will be evaluated for
conformity to the genotype/phenotype of the vaccine
strain.

    Bacteriology: Stools and rectal swabs will be
inoculated into selenite-cystine broth. Stools must be

30 processed within 48 hours. After overnight incubation at
37°C, subcultures will be made onto XLT-4 agar. Colonies
which appear consistent with *Salmonella* will be processed

through API-20 system of identification and confirmation made by a agglutenation with *S. typhi* O, H, and Vi antisera. These isolates will be saved at -70°C in 5% glycerol-1% peptone for further analysis (e.g., for the

5   presence of plasmids, for absence or presence of specific DNA sequences using PCR, or for Southern blotting with gene probes for cloned genes).

Blood cultures (10 ml) will be inoculated in 50 ml Septacheck bottles. Positive cultures are analyzed and

10   saved as described above.

Immunology: Sera specimens will be tested for IgA, IgM and IgG to *S. typhi* O, H and Vi antigens measured by ELISA. H antibody will also be measured by Widal tube agglutination using *S. virginia* as antigen (*S. manhatten*

15   also shares the identical flagellar antigen as *S. typhi* but not somantic antigen). Peripheral blood mononuclear cells will be collected and separated for antibody secreting cell (ASC) assays employing ELISPOT for cells producing antibody to *Salmonella* antigens. Lymphocytes

20   that secret IgG, IgA, or IgM against *S. typhi* O, H, or Vi antigens will be measured.

PCR: The *Salmonella invA* gene segment will be amplified by polymerase chain reaction to confirm the presence or absence of *Salmonella typhi* in blood

25   specimens. The *invA* sequence is unique to *Salmonella* (Galan and Curtiss, 1991) and is diagnostic for the presence of invasive *Salmonella* by PCR methods (Rahn et al., 1992).

Excretion of the Vaccine Strain: It is expected that

30   excretion of the vaccine strain would cease within 1 week after a dose of vaccine. If excretion continues for 7 or more days, the volunteer who continues to excrete is

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given a dose of ciprofloxacin (700 mg every 12 hours).

Negative cultures for a ≥ 2 consecutive days are required

for discharge.

5   Example 16

This Example illustrates the potential use of

attenuated RpoS⁺ *Salmonella enterica* of various serotypes

for intranasal administration to elicit superior mucosal

and systemic immune responses.   Such candidate vaccines

10   can also be administered orally, conjuntivally, or

rectally.

The *Salmonella enterica* serotypes are, preferably,

attenuated with known attenuation approaches such as by

generating deletion mutations in a *pab* gene, a *pur* gene,

15   an *aro* gene, *asd*, a *dap* gene, *nadA*, *pncB*, *galE*, *pmi*, *fur*,

rpsL, *ompR*, *htrA*, *hemA*, *cdt*, *cya*, *crp*, *phoP*, *phoQ*, *rfc*,

poxR, *galU* or in a combination of these genes.

Furthermore, the microbes would have an RpoS⁺ phenotype as

determined by the catalase test or the glycogen synthesis

20   test as described in Example 4.   The RpoS⁺ attenuated

Salmonella enterica strains could be of the serotypes

Typhi, Paratyphi A, Paratyphi B, Paratyphi C,

Typhimurium, Enteritidis, Dublin, or Choleraesuis.   In

the wide host range *S. enterica* serotypes, Typhimurium

25   and Enteritidis, and in the more host-adapted serotypes,

Dublin and Choleraesuis, it is desirable that they

possess the *Salmonella* virulence plasmid which enhances

their immunogenicity due to more rapid growth in

intracellular in vivo environments (Gulig in *Escherichia*

30   *coli* and *Salmonella*, Vol 2, F. Neidhardt et al., Editor,

American Society for Microbiology, Washington DC, pp.

2774-2787, 1996).

Study design for testing safety and efficacy in humans are as described in Example 15 above except for the serotype of *Salmonella* to be administered and the nasal route of immunization. Table 15 lists parental

5 vaccine vector strains with differing serotypes and their test results for catalase and glycogen synthesis to indicate the RpoS$^+$ phenotype.

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Table 15.

Determination of RpoS Phenotype of Bacterial Strains.

Bacterial species	Glycogen Accumulation/ Biosynthesis[a]	Catalase Activity
χ3246 *Salmonella choleraesuis*	−	+
χ3759 *Salmonella enteritidis*	+	+
χ3841 *Salmonella infantis*	+	+
χ4952 *Salmonella pullorum*	+	+
χ4821 *Salmonella dublin*	n.g.	+
χ8274 *Salmonella typhimurium* 14028s	+	+
χ8219 *Salmonella paratyphi* A	+	−
χ8436 *Salmonella paratyphi* C	+	+
χ8437 *Salmonella sendai*	+	+
Shigella flexneri 2a 2457T	n.g.	+

[a]n.g.-no growth on Q-3 medium.

Note that *S choleraesuis* χ3246 is unable to synthesize glycogen, however, it tests as RpoS[+] by the catalase test. *S. paratyphi* A, χ8219 synthesizes glycogen indicating an RpoS[+] phenotype, but lacks the catalase regulated by the *rpoS* gene. Any of these strains can be attenuated by the methods described in Examples 2 and 3 and further modified with the *asd* mutation for use of an Asd[+] vector encoding for a foreign antigen such as the pYA3167 specifying the hepatitis B virus core pre-S1/preS-2 fusion as described in Example

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11. All of these strains display the RpoS[+] phenotype.

The other difference between the procedures described in Example 15 is the route of immunization. Intranasal immunization can be achieved by administration of nose drops containing the vaccine strain at a suitable dose while the individual is lying prone with head turned back. Alternatively, intranasal immunization can be achieved by aerosolization into the nostrils with a nebulizer. The dose administered is determined by the number of squirts.

Other routes of administration can also be tested. For example, rectal immunization can be achieved by the procedures described by Nardelli-Haefliger et al. (Infect Immun, 1996). Intraconjunctival immunization can be achieved by administration of eye drops. All of the monitoring and well being of immunized subjects and for the elicitation of appropriate immune responses are as described in Example 15.

Example 17

This example illustrates methods for preparation of RpoS[+] *Salmonella, Shigella/Escherichia* and *Salmonella/Escherichia* hybrids for use in delivering DNA vaccine vectors to a human.

Circular plasmid DNA encoding antigens of various pathogens can be introduced into animal hosts to stimulate the induction of immunity to the pathogen from which the antigen gene was derived (Ullmer et al., *ASM News 62*:476-479, 1996; Ullmer et al., *Curr. Opin. Immunol 8*:531-536, 1996; Whalen, *Emerg. Infect. Dis. 2*:168-175, 1996; Robinson, *Vaccine 15*:785-787, 1997). DNA vaccines make use of expression systems such that the genetic

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information specifying the antigen of some pathogen is expressed by the immunized hosts using host machinery for transcription and translation. Initially, DNA vaccines were administered by injection into muscle tissue, but other injection sites have also been used. Most recently, DNA vaccines have been administered using particle guns to accelerate entry of DNA-coated gold beads into skin or mucosal tissues. The DNA vaccine vbectors are propagated in and isolated from recombinant *E.coli* strains grown in fermentors.

Sizemore et al. (*Science, 270:299-302, 1995; Vaccine 15:804-807, 1997*) described the use of a *Shigella flexneri* 2a strain with a Δ*asd* mutation that harbored a DNA vaccine vector engineered to express *E. coli* β-galactosidase. The *Shigella* strain was attenuated due to the Δ*asd* mutation which causes death due to absence of diaminopimelic acid upon invasion into eukaryotic cells. The strain was able to deliver the DNA vaccine vector intracellularly after attachment to, invasion into and lysis within the cytoplasm of eukaryotic cells in culture or within immunized mice. More recently, others have used *S. typhimurium* strains possessing a DNA vaccine vector and caused to lyse by spontaneous means (Powell et al., WO96/34631, 1996; Pasenal et al., *Behring. Inst. Mitt. 98:143-152, 1997*; Darji et al., *Cell 91:765-775, 1997*). In cases in which lysis was spontaneous, it was necessary that the bacterial strain possess one or more deletion mutations rendering the strain attenuated. *Shigella, Salmonella* and invasive *E. coli* are known to have a much enhanced ability to attach to and invade M cells overlying the GALT rather than to attach to and invade intestinal epithelial cells (enterocytes).

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Delivery of foreign antigens or the production of foreign antigens within the NALT, BALT, CALT and GALT which all have an M cell layer leads to induction of mucosal immune responses as well as systemic immunity. Because mucosal immune responses are protective against the vast majority of infectious disease agents that colonize on or invade through a mucosal surface, it would be expected that DNA vaccine vectors could thus be delivered by RpoS$^+$ *Salmonella, Shigella, Escherichia* or hybrids between any two of these genera. These microbes would have a superior ability to attach to and invade the M cells overlying the lymphoid tissues of the NALT, CALT, BALT and GALT. Because both oral and intranasal immunization with RpoS$^+$ microbes increase the immune response, it would be expected that attenuated bacterial DNA vaccine vector strains displaying an RpoS$^+$ phenotype will give an increased immune response when administered intranasally or perorally and presumably by other routes that stimulate mucosal immune responses.

We have used derivatives of the DNA vaccine vector pCMVβ to express foreign antigens. pCMVβ possesses the pUC origin of replication for propagation in *E. coli*, a β-lactamase gene to confer resistance to ampicillin, promoters and enhancers from CMV and SV40 viruses and an SV40 sequence to achieve polyadenylation of the transcribed mRNA (MacGregor et al., *Nucleic Acid Res.* *17*:1265, 1989). pCMVβ contains the coding sequence for *E. coli* β-galactosidase which has been used as a test antigen in several studies. The *lacZ* gene encoding β-galactosidase can be easily removed with substitution of DNA encoding a diversity of antigens, especially from viral, fungal and parasitic pathogens. Since introducing

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antibiotic resistance genes as parts of vaccines into
immunized animal and human hosts continues to be a
concern, we have substituted the *S. typhimurium asd* gene
for the ampicillin-resistance gene in pCMVβ to yield
pCMVβ-*asd* (Figure 14). This enables the use of an *E.
coli* host that has a Δ*asd* mutation to yield a balanced-
lethal host-vector system that can be propagated in the
fermentor in the absence of added costly antibiotics that
could also potentially contaminate the purified DNA
vaccine vector. Furthermore, the *S. typhimurium asd* gene
possesses two natural CpG sequences (Kreig, *J. Lab. Clin.
Med 128*:128-133, 1996) that strongly enhance the
immunogenicity of the DNA vaccine vector. Such sequences
are absent in the kanamycin-resistance gene that is now
often used in lieu of the ampicillin-resistance gene in
DNA vaccines. The use of the *S. typhimurium asd* gene in
such DNA vaccine vectors is described in U.S. Patent no.
5,840,483.

Commercial Utility:

The bacterial strains provided herein are directly
and indirectly suitable for production of immunogenic
compositions, including vaccines, to prevent diseases
caused by various bacterial, viral, fungal protazoal
pathogens. These carrier bacterial strains which can
be, for example, *S.* typhi strains, all have an RpoS[+]
phenotype, and can serve as carriers for delivering to
target tissues, heterologous proteins or nucleic acid
molecules for expression of gene products. The microbes
are not only attenuated, but also show high
immunogenicity because of an improved ability to colonize
lymphoid tissue compared to previously used recombinant

attenuated bacteria. The present strains are also useful
as carrier microorganisms for the production of
expression products encoded on recombinant genes in the
bacterial cells. In addition, the strains which can be
used with enhanced safety and improved immunogenicity are
highly effective in the production of antibodies against
recombinant antigens which can be expressed in the
attenuated, immunogenic bacteria.

Deposit:

    The following strains and plasmid are on deposit
under the terms of the Budapest Treaty, with the American
Type Culture Collection, 12301 Parklawn Drive, Rockville,
MD. The accession number indicated was assigned after
successful viability testing, and the requisite fees were
paid. Access to the cultures and plasmid will be
available during pendency of the patent application to
one determined by the Commissioner to be entitled thereto
under 37 CFR 1.14 and 35 USC 122. All restriction on
availability of the cultures and plasmid to the public
will be irrevocably removed upon the granting of a patent
based upon the application. Moreover, the designated
deposits will be maintained for a period of thirty (30)
years from the date of deposit, or for five (5) years
after the last request for the deposit, or for the
enforceable life of the U.S. patent, whichever is longer.
Should a culture or plasmid become nonviable or be
inadvertently destroyed, or, in the case of plasmid-
containing strains, lose its plasmid, it will be replaced
with a viable culture. The deposited materials mentioned
herein are intended for convenience only, and are not
required to practice the present invention in view of the

description herein, and in addition, these materials are
incorporated herein by reference.

Deposit	Deposit Date	ATCC No.
Strains:		
MGN-1191	November 14, 1997	202054
MGN-1256	November 14, 1997	202053
χ8280	November 14, 1997	202055
χ8281	November 14, 1997	202056
χ8438	November 1998	
Plasmid:		
pYA3433	November 14, 1997	209462

In view of the above, it will be seen that the
several advantages of the invention are achieved and
other advantageous results attained.

As various changes could be made in the above
methods and compositions without departing from the scope
of the invention, it is intended that all matter
contained in the above description and shown in the
accompanying drawings shall be interpreted as
illustrative and not in a limiting sense.

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<u>What is Claimed is:</u>

1. A method for delivery of a desired gene product to a human comprising:

(a) selecting for a strain of bacteria having (i) as RpoS$^+$ phenotype, (ii) one or more inactivating mutations which render the strain attenutated, and (iii) a recombinant gene encoding the desired gene product; and

(b) administering the strain to the human.

2. The method according to claim 1 wherein selecting a strain of bacteria comprises selecting a strain of *Salmonella*.

3. The method according to claim 2 wherein selecting a strain of *Salmonella* comprises selecting a strain of *S. typhi*.

4. The method according to claim 3 wherein the strain of *S. typhi* comprises an inactivating mutation in a *pab* gene, a *pur* gene, an *aro* gene, *asd*, a *dap* gene, *nadA, pncB, galE, pmi, fur, rpsL, ompR, htrA, hemA, cdt, cya, crp, phoP, phoQ, rfc, poxR, galU* or a combination thereof.

5. The method according to claim 4 wherein the *S. typhi* is derived from MGN-1191.

6. The method according to claim 4 wherein the strain of *S. typhi* is capable of expressing a recombinant gene that encodes a product from a pathogen to said human.

7. The method according to claim 6 wherein the pathogen is a virus, bacterium, protozoan, parasite or fungus.

8. The method according to claim 7 wherein the *S. typhi* is χ8281.

9. The method according to claim 4 wherein the strain of *S. typhi* is capable of expressing a recombinant gene that encodes a product capable of suppressing, modulating, or augmenting an immune response in said human.

10. The method according to claim 4 wherein the strain of *S. typhi* contains at least one recombinant gene encoding an auto-antigen.

11. The method according to claim 10 wherein the auto-antigen is a gamete-specific antigen.

12. The method according to claim 4 wherein the second recombinant gene encodes an allergen to said human.

13. A method for delivery of a desired gene product to a human comprising administering to the human a live attenuated strain of bacteria having (a) an RpoS$^+$ phenotype, (b) a recombinant *rpoS$^+$* gene, (c) one or more inactivating mutations which render said microbe attenuated and (c) a second recombinant gene encoding the desired gene product.

14. The method according to claim 13 wherein administering a strain of bacteria comprises administering a strain of *Salmonella*.

15. The method according to claim 14 wherein administering a strain of *Salmonella* comprises administering a strain of *S. typhi*.

16. The method according to claim 15 wherein the strain of *S. typhi* is derived from χ8438.

17. The method according to claim 15 wherein the strain of *S. typhi* comprises an inactivating mutation in a *pab* gene, a *pur* gene, an *aro* gene, *asd*, a *dap* gene, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*, *hemA*, *cdt*,

5   *cya, crp, phoP, phoQ, rfc, poxR, galU* or combination

thereof.

18. The method according to claim 17 wherein the

second recombinant gene encodes a gene product from a

pathogen to said human.

19. The method according to claim 18 wherein the

pathogen is a virus, bacterium, protozoan, parasite or

fungus.

20. The method according to claim 17 wherein the

second recombinant gene encodes a product capable of

suppressing, modulating, or augmenting an immune response

in said human.

21. The method according to claim 17 wherein the

second recombinant gene encodes an auto-antigen.

22. The method according to claim 21 wherein the

auto-antigen is a gamete-specific antigen.

23. The method according to claim 17 wherein the

recombinant gene encodes an allergen to said human.

24. A method for producing a strain of carrier

microbes for delivery of a desired gene product to a

human comprising in any order the steps of:

(a) selecting for a strain of bacteria having an

5   RpoS$^+$ phenotype by performing a test to determine the RpoS

phenotype of the strain;

(b) producing one or more inactivating mutations

which render the strain attenuated; and

(c) introducing into the strain a recombinant gene

10   encoding a desired gene product.

25. The method according to claim 24 wherein

selecting for a strain of bacteria comprises selecting

for a strain of *Salmonella*.

26. The method according to claim 25 wherein selecting for a strain of *Salmonella* comprises selecting for a strain of *S. typhi*.

27. The method according to claim 26 wherein the strain of *S. typhi* comprises an inactivating mutation in a *pab* gene, a *pur* gene, an *aro* gene, *asd*, a *dap* gene, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*, *hemA*, *cdt*, *cya*, *cya*, *phoP*, *phoQ*, *rfc*, *poxR*, *galU* or combination thereof.

28. The method according to claim 27 wherein the *S. typhi* is derived from MGN-1191.

29. The method according to claim 27 wherein the strain of *S. typhi* contains a recombinant gene encoding a gene product from a pathogen to said human.

30. The method according to claim 29 wherein the pathogen is a virus, bacterium, protozoan, parasite or fungus.

31. The method according to claim 30 wherein the *S. typhi* is χ8281.

32. The method according to claim 27 wherein the strain of *S. typhi* contains a recombinant gene encoding a product capable of suppressing, modulating, or augmenting an immune response in said human.

33. The method according to claim 27 wherein the strain of *S. typhi* contains at least one recombinant gene encoding an auto-antigen.

34. The method according to claim 33 wherein the auto-antigen is a gamete-specific antigen.

35. The method according to claim 27 wherein the recombinant gene encodes an allergen to said human.

36. A method for producing carrier microbes for delivery of a desired gene product to a human comprising

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generating a strain of bacteria having (a) an RpoS$^+$
phenotype, (b) a recombinant $rpoS^+$ gene, (c) one or more

5   inactivating mutations which render said microbe
attenuated and (c) a second recombinant gene encoding the
desired gene product.

      37. The method according to claim 36 wherein
generating a strain of bacteria comprises generating a
strain of *Salmonella*.

      38. The method according ot claim 37 wherein
generating a strain of *Salmonella* comprises generating a
strain of *S. typhi*.

      39. The method according to claim 38 wherein the
strain of *S. typhi* is derived from χ8438.

      40. The method according to claim 38 wherein the
strain of *S. typhi* comprises an inactivating mutation in
a *pab* gene, a *pur* gene, an *aro* gene, *asd*, a *dap* gene,
nadA, pncB, galE, pmi, fur, rpsL, ompR, htrA, hemA, cdt,

5   *cya, crp phoP, phoQ, rfc, poxR, galU* or combination
thereof.

      41. The method according to claim 39 wherein the
second recombinant gene encodes a gene product from a
pathogen to said human.

      42. The method according to claim 41 wherein the
pathogen is a virus, bacterium, protozoan, parasite or
fungus.

      43. The method according to claim 39 wherein the
second recombinant gene encodes a product capable of
suppressing, modulating, or augmenting an immune response
in said human.

      44. The method according to claim 39 wherein the
second recombinant gene encodes an auto-antigen.

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45. The method according to claim 39 wherein the auto-antigen is a gamete-specific antigen.

46. The method according to claim 39 wherein the recombinant gene encodes an allergen to said human.

47. A carrier microbe for the delivery of a desired gene product to a human comprising a live attenuated bacteria having (a) an RpoS⁺ phenotype, (b) a recombinant *rpoS⁺* gene, (c) one or more inactivating

5   mutations which render said microbe attenuated and (c) a second recombinant gene encoding the desired gene product.

48. A carrier microbe according to claim 47 wherein the bacteria comprises a *Salmonella*.

49. A carrier microbe according to claim 48 wherein the *Salmonella* comprises an *S. typhi*.

50. The method according to claim 49 wherein the strain of *S. typhi* is derived from χ8438.

51. The carrier microbe according to claim 49 wherein the attenuated *S. typhi* comprises an inactivating mutation in a *pab* gene, a *pur* gene, an *aro* gene, *asd*, a *dap* gene, *nadA*, *pncB*, *galE*, *pmi*, *fur*, *rpsL*, *ompR*, *htrA*,

5   *hemA*, *cdt*, *cya*, *crp*, *phoP*, *phoQ*, *rfc*, *poxR*, *galU* or combination thereof.

52. The carrier microbe according to claim 51 wherein the second recombinant gene encodes a gene product from a pathogen to said human.

53. The carrier microbe according to claim 52 wherein the pathogen is a virus, bacterium, protozoan, parasite or fungus.

54. The carrier microbe according to claim 51 wherein the second recombinant gene encodes a product

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capable of suppressing, modulating, or augmenting an
immune response in said human.

55. The carrier microbe according to claim 51
wherein the second recombinant gene encodes an auto-
antigen.

56. The carrier microbe according to claim 55
wherein the auto-antigen is a gamete-specific antigen.

57. The carrier microbe according to claim 51
wherein the recombinant gene encodes an allergen to said
human.

58. A vaccine for immunization of a human
comprising a live attenuated strain of bacteria having
(a) an RpoS$^+$ phenotype, (b) a recombinant rpoS$^+$ gene, (c)
one or more inactivating mutations which render said
5   microbe attenuated and (c) a second recombinant gene
encoding the desired gene product.

59. A vaccine according to claim 58 wherein the
bacteria comprises a Salmonella.

60. A vaccine according to claim 59 wherein the
Salmonella comprises an S. typhi.

61. The method according to claim 60 wherein the
strain of S. typhi is derived from χ8438.

62. The vaccine according to claim 60 wherein the
strain of S. typhi comprises an inactivating mutation in
a pab gene, a pur gene, an aro gene, asd, a dap gene,
nadA, pncB, galE, pmi, fur, rpsL, ompR, htrA, hemA, cdt,
5   cya, crp, phoP, phoQ, rfc, poxR, galU or combination
thereof.

63. The vaccine according to claim 62 wherein the
second recombinant gene encodes a gene product from a
pathogen to said human.

64. The vaccine according to claim 63 wherein the pathogen is a virus, bacterium, protozoan, parasite or fungus.

65. The vaccine according to claim 62 wherein the second recombinant gene encodes an auto-antigen.

66. The vaccine according to claim 65 wherein the auto-antigen is a gamete-specific antigen.

67. The vaccine according to claim 62 wherein the second recombinant gene encodes an allergen to said human.

68. The vaccine according to claim 58 wherein said attenuated strain of *S. typhi* is in a pharmaceutically acceptable carrier.

69. A genetically engineered cell comprising a live attenuated strain of bacteria having (a) an RpoS$^+$ phenotype, (b) a recombinant *rpoS*$^+$ gene, (c) one or more inactivating mutations which render said microbe attenuated and (c) a second recombinant gene encoding the desired gene product.

70. The genetically engineered cell according to claim 69 wherein the strain of bacteria comprises a strain of *Salmonella*.

71. The genetically engineered cell according to claim 70 wherein the strain of *Salmonella* comprises a strain of *S. typhi*.

72. The method according to claim 71 wherein the strain of *S. typhi* is derived from χ8438.

73. The genetically engineered cell according to claim 71 wherein the attenuated strain of *S. typhi* comprises an inactivating mutation in a *pab* gene, a *pur* gene, an *aro* gene, *asd*, a *dap* gene, *nadA*, *pncB*, *galE*,

5 *pmi, fur, rpsL, ompR, htrA, hemA, cdt, cya, crp, phoP,*

phoQ, rfc, poxR, galU or combination thereof.

74. The genetically engineered cell according to
claim 73 wherein the second recombinant gene encodes a
gene product from a pathogen to said human.

75. The genetically engineered cell according to
claim 74 wherein the pathogen is a virus, bacterium,
protozoan, parasite or fungus.

76. The genetically engineered cell according to
claim 73 wherein the second recombinant gene encodes a
product capable of suppressing, modulating, or augmenting
an immune response in said human.

77. The genetically engineered cell according to
claim 73 wherein the second recombinant gene encodes an
auto-antigen.

78. The genetically engineered cell according to
claim 77 wherein the auto-antigen is a gamete-specific
antigen.

79. The genetically engineered cell according to·
claim 73 wherein the recombinant gene encodes an allergen
to said human.

80. A method for preparing a vaccine comprising
mixing genetically engineered cells according to claim 69
with a pharmaceutically acceptable carrier.

81. A method for delivery of a desired gene
product to a human comprising administering to the human
a live attenuated strain of bacteria having (a) a
recombinant virulence gene which is capable of expressing

5 a gene product that facilitate invasion and colonization
of the gut associated lymphoid tissues, (b) one or more
inactivating mutations which render said microbe

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attenuated and (c) a second recombinant gene encoding the desired product.

82. The method according to claim 81 wherein the strain of bacteria is a strain of *Salmonella*.

83. The method according to claim 82 wherein the strain of *Salmonella* is a strain of *S. typhi*.

84. A genetically engineered cell comprising a strain of live atttenuated bacteria having (a) a recombinant virulence gene which is capable of expressing a gene product that facilitates invasion and colonization of the gut associated lymphoid tissues, (b) one or more inactivating mutations which render said microbe attenuated and (c) a second recombinant gene encoding the desired product.

85. The genetically engineered cell according to claim 84 wherein the bacteria comprise *Salmonella*.

86. The genetically engineered cell according to claim 85 wherein the *Salmonella* comprise *S. typhi*.

87. A method for assessing the immunogenicity of a bacteria comprising determining the RpoS phenotype of said bacteria wherein the presence of an RpoS$^+$ phenotype indicates increased immunogenicity compared to an isogenic bacteria having an RpoS$^-$ phenotype.

88. The method of claim 87 wherein the bacteria comprise *Salmonella*.

89. The method of claim 88 wherein the *Salmonella* comprise *S. typhi*.

90. The method of claim 89 wherein the RpoS phenotype is determined by assessing one or both of catalase activity and glycogen biosynthesis activity of the *S. typhi*.
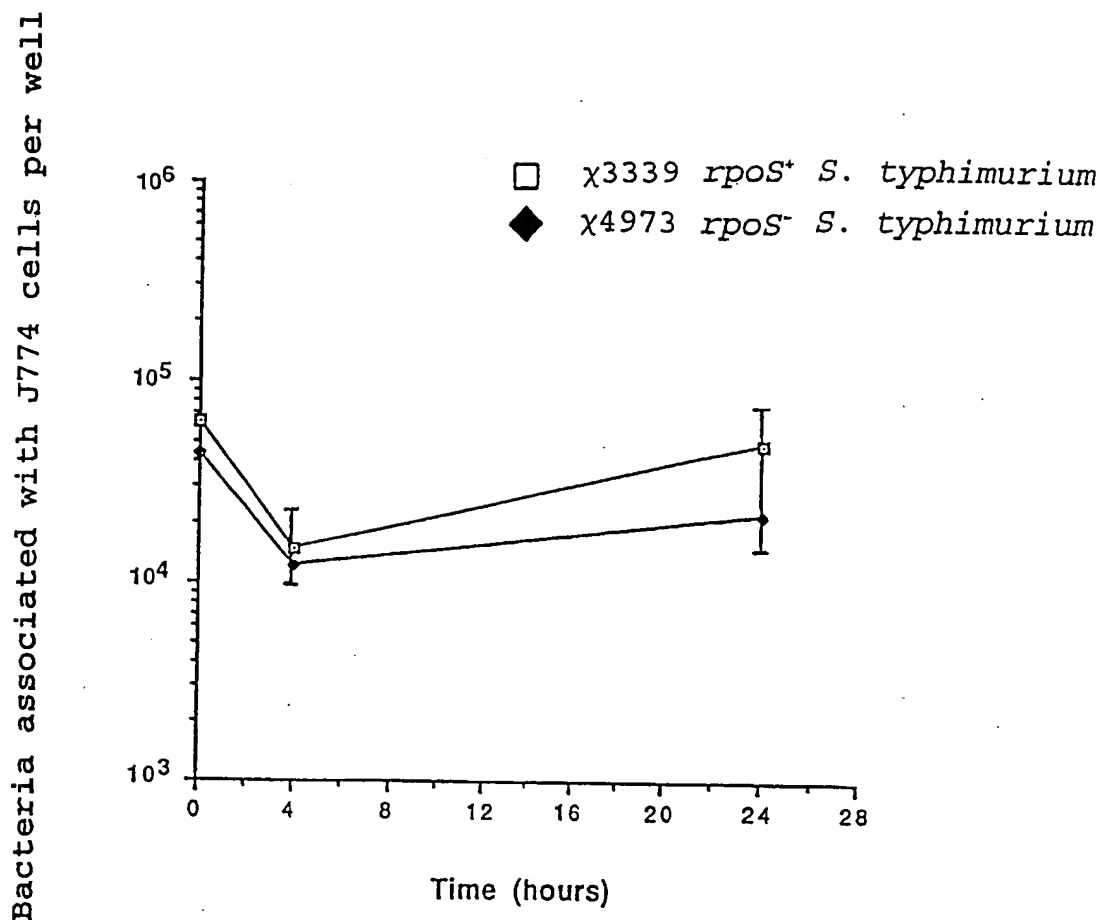
Figure 1

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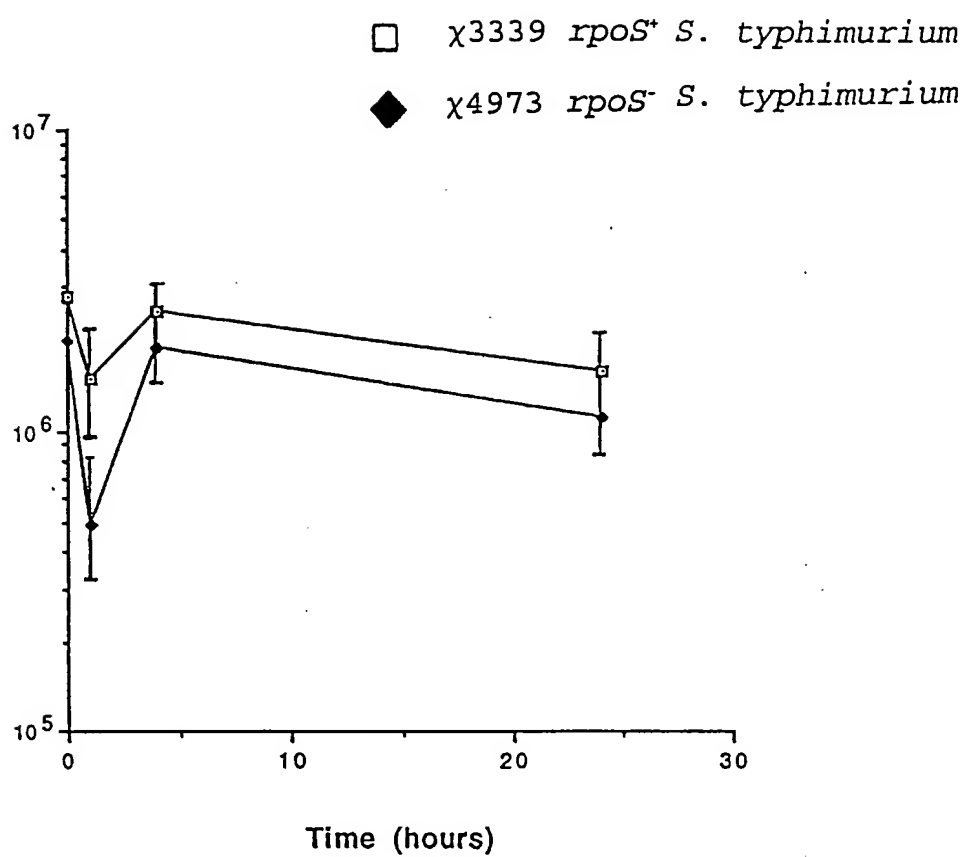
Figure 2

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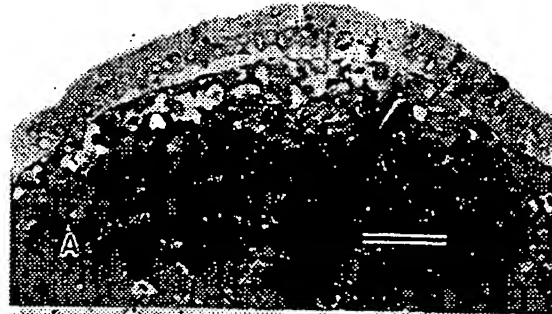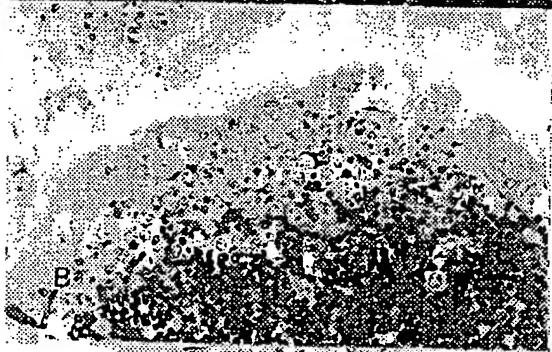
Figure 3A

Figure 3B

Figure 3C

Figure 3D
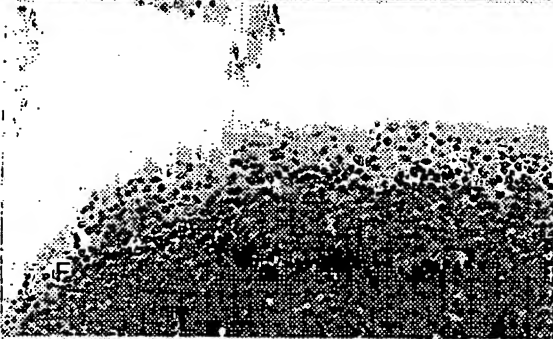
Figure 3E

Figure 3F
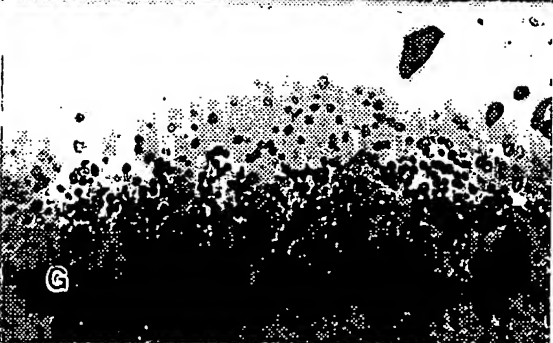
Figure 3G
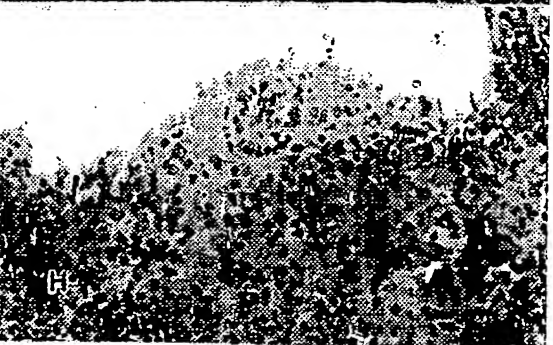
Figure 3H

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Figure 4A
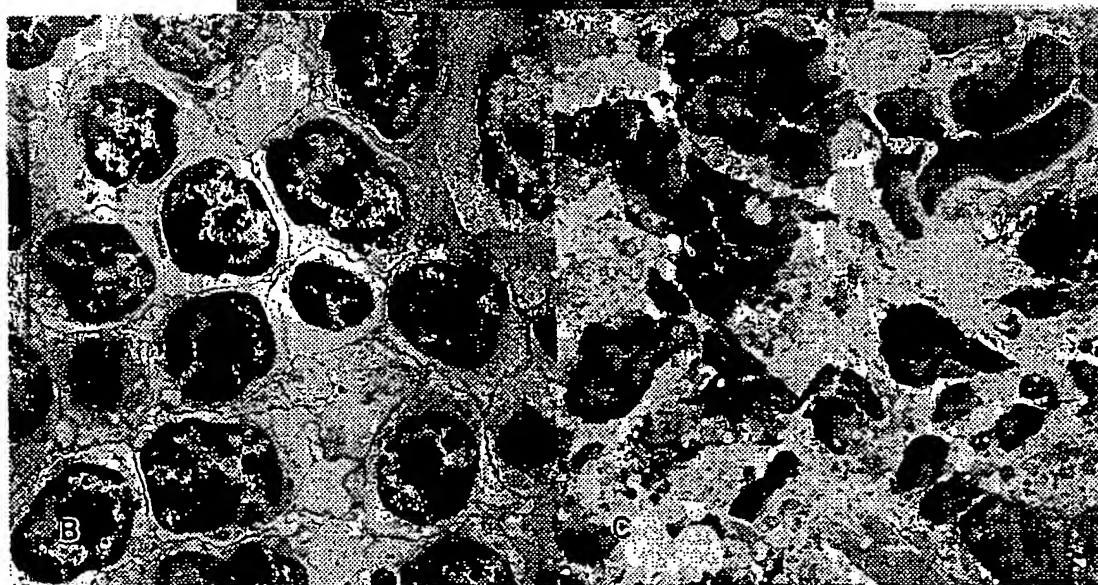
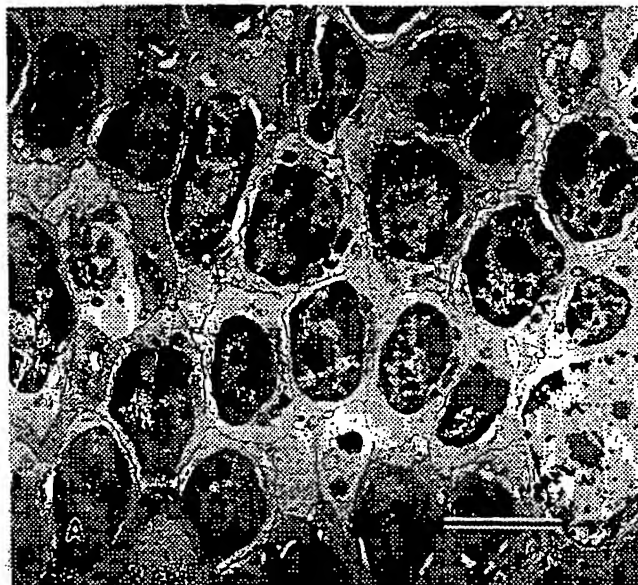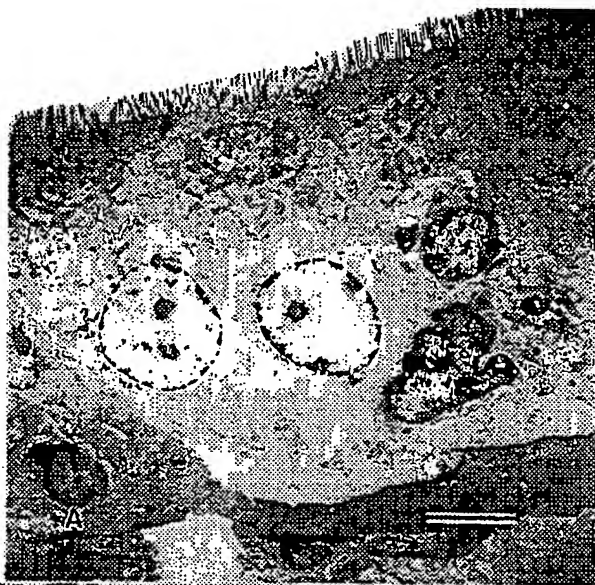Figure 4B   Figure 4C
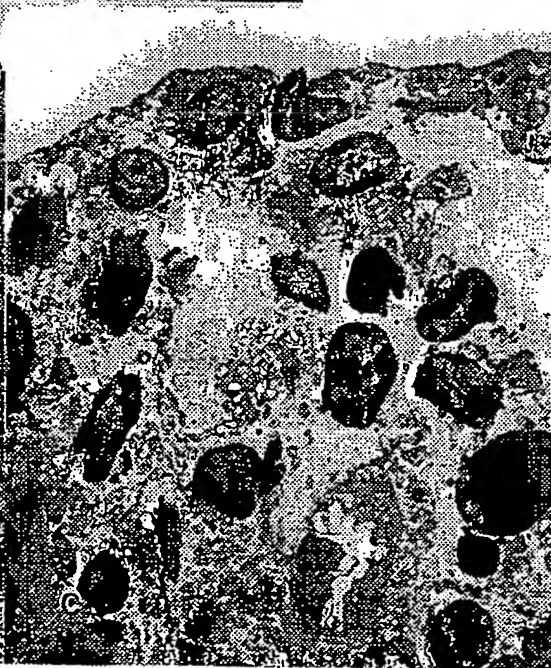
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Figure 5A
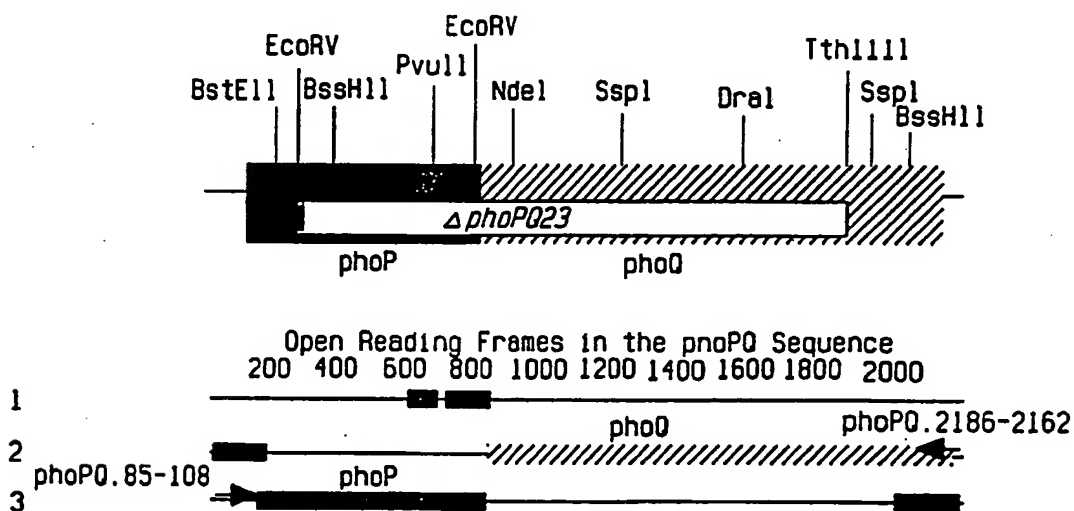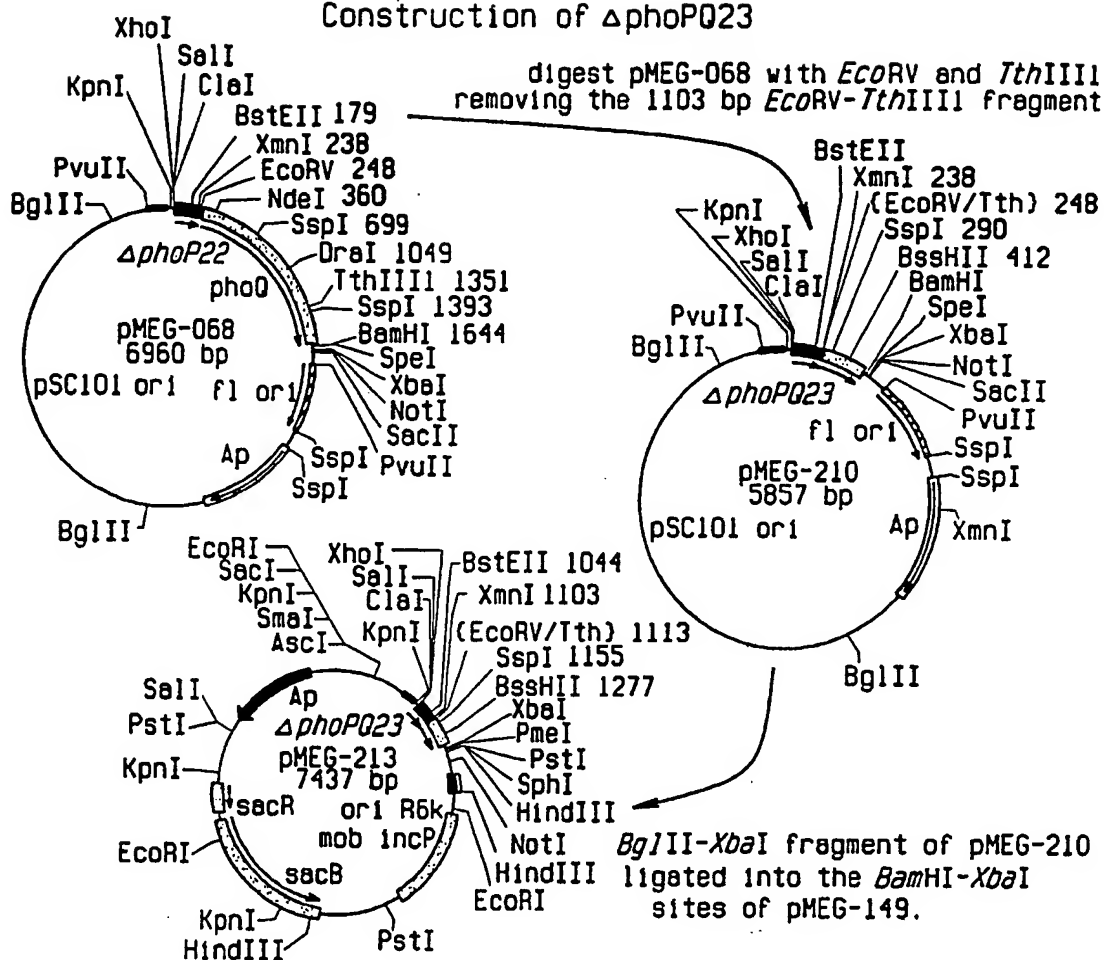

Figure 5B   Figure 5C

Construction of ΔphoPQ23

digest pMEG-068 with *EcoRV* and *Tth*IIII
removing the 1103 bp *EcoRV-Tth*IIII fragment

*Bgl*II-*Xba*I fragment of pMEG-210
ligated into the *Bam*HI-*Xba*I
sites of pMEG-149.

Open Reading Frames in the pnoPQ Sequence

FIG.6

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Construction of Defined Asd Deletion Vector
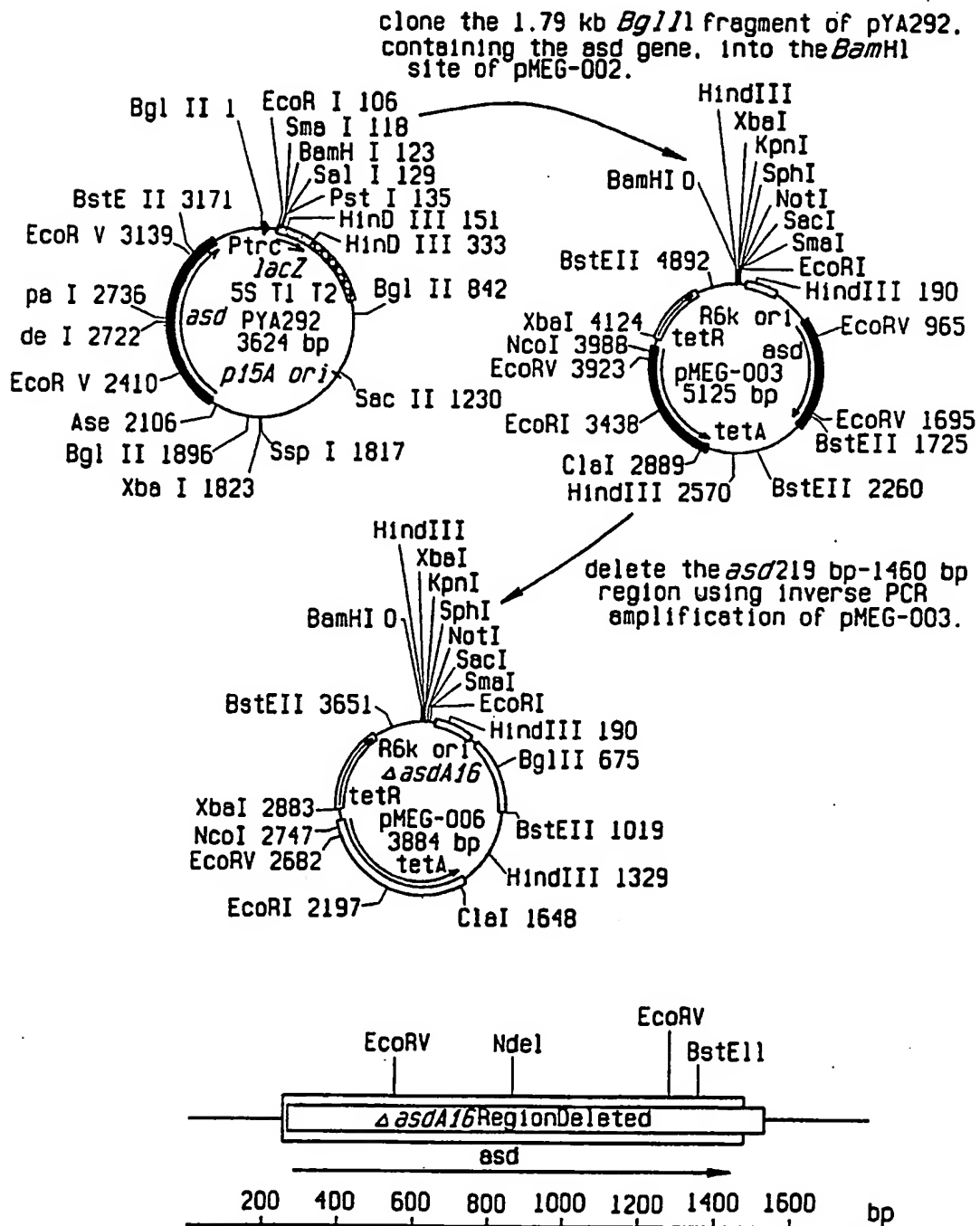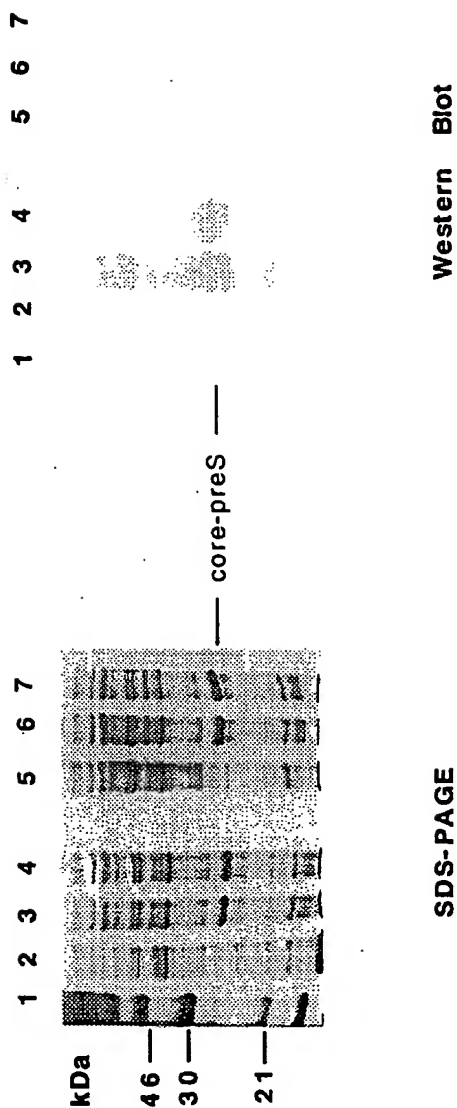
clone the 1.79 kb *BglII* fragment of pYA292.
containing the asd gene. into the *Bam*Hl
site of pMEG-002.

FIG. 7

Western   Blot

Figure 8B

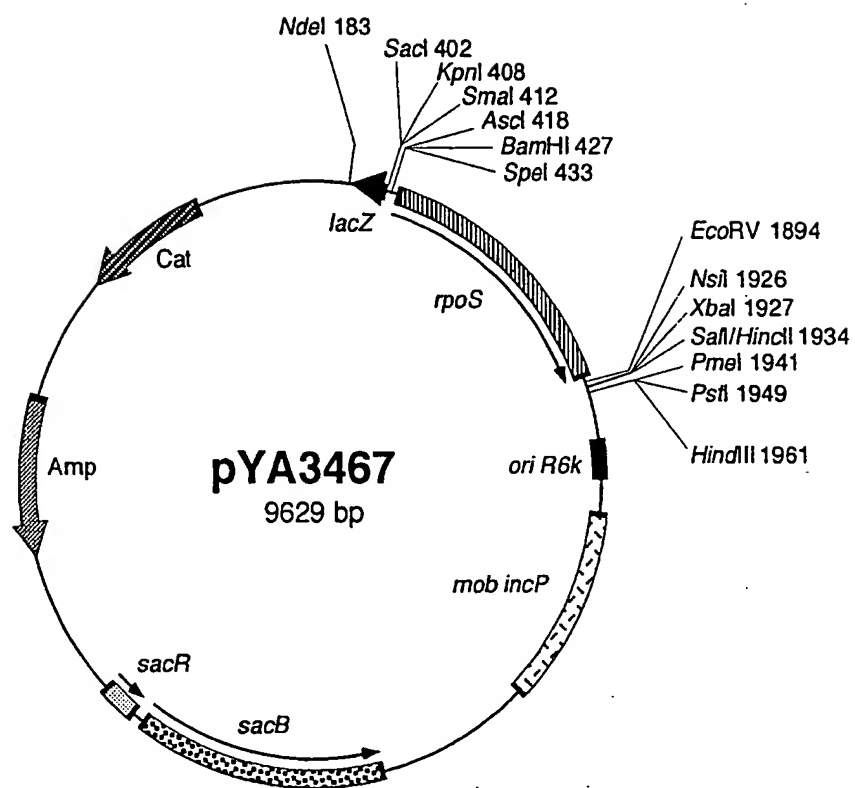core-preS ———

SDS-PAGE

Figure 8A

kDa

46 —
30 —

21 —

A.

Figure 9A

B.
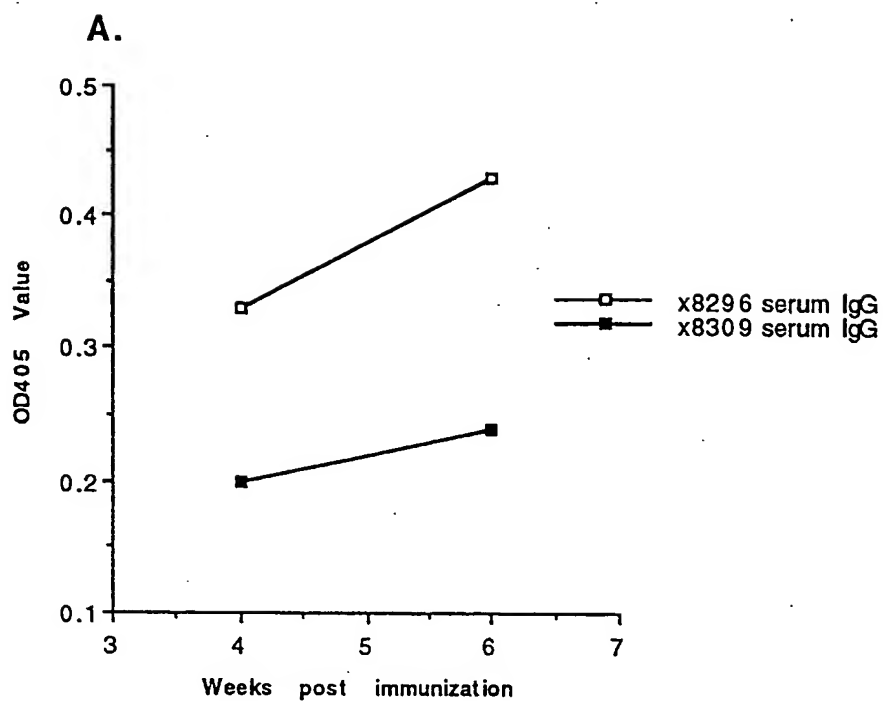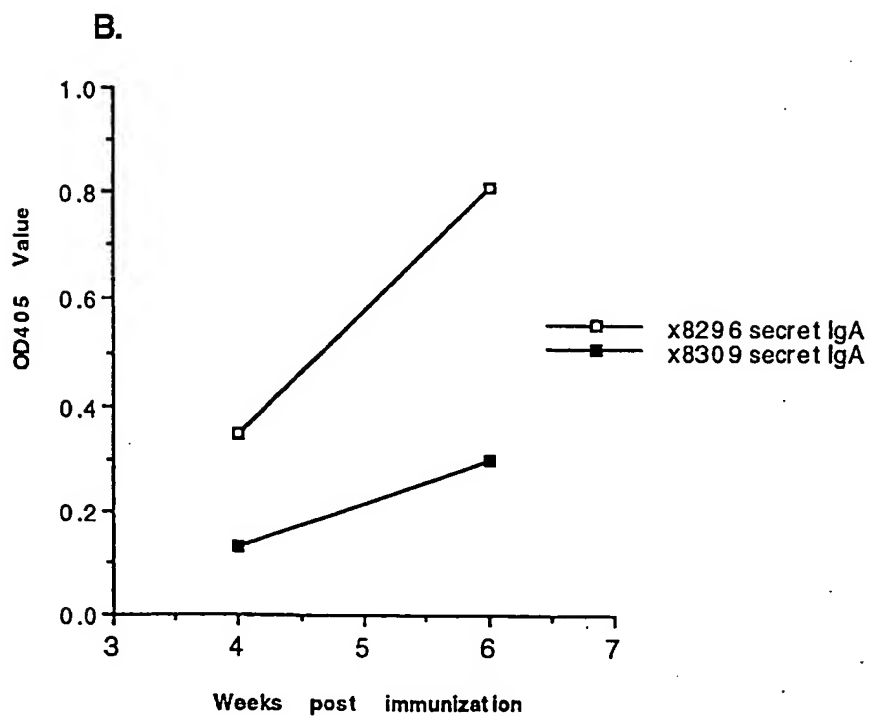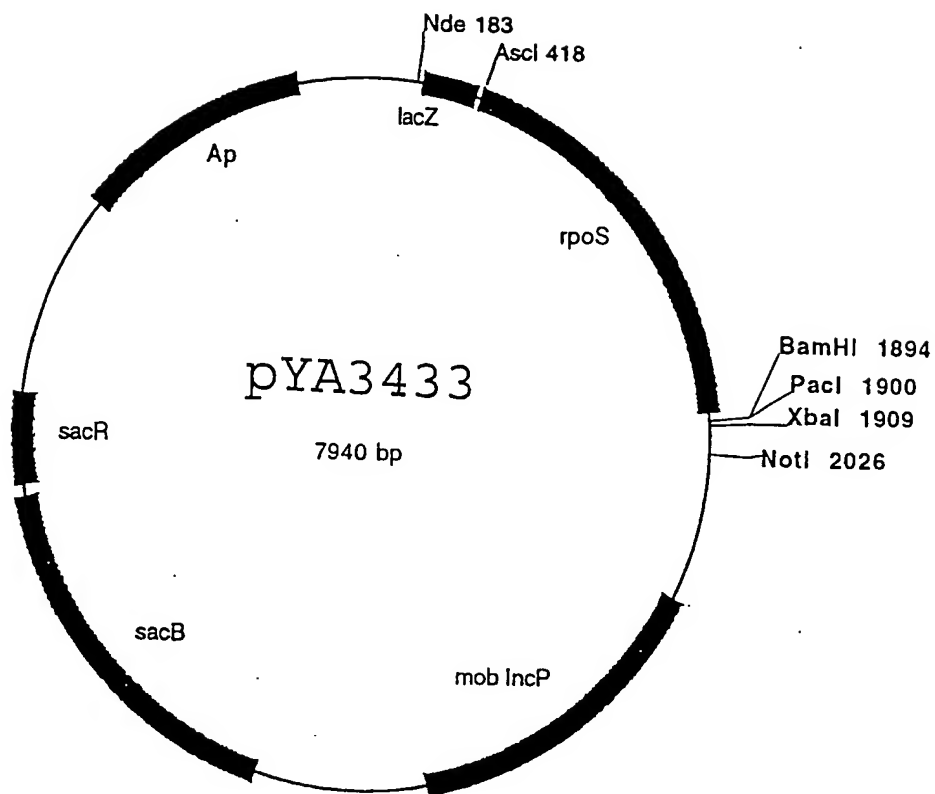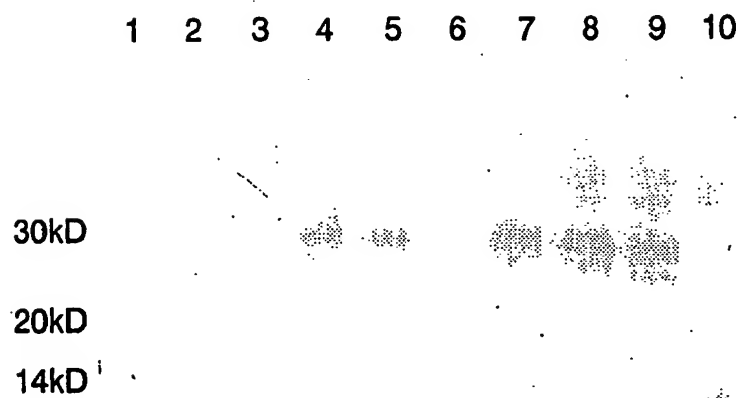
Figure 9B

Figure 10

Figure 11

Figure 12

Figure 13

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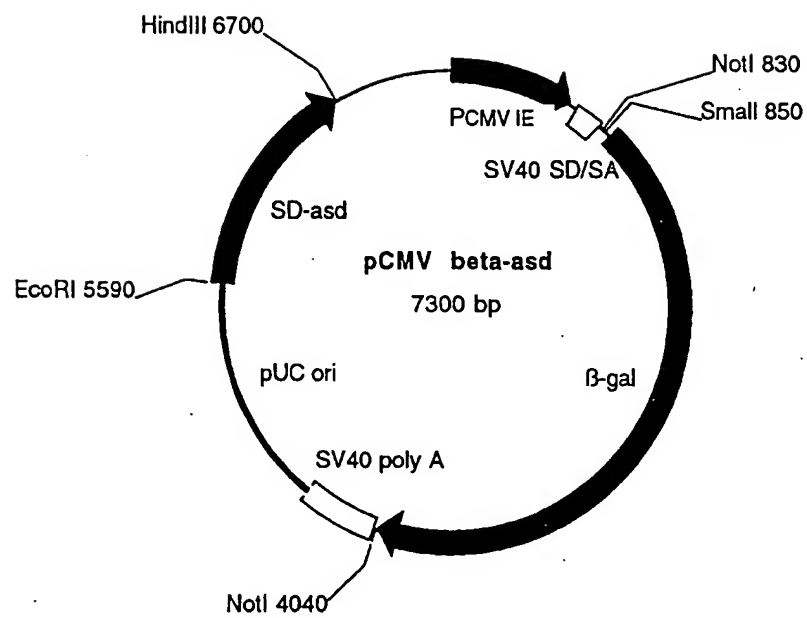
Figure 14

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   A61K48/00   C12N1/22   A61K39/112

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CURTISS R 3D ET AL: "Avirulent Salmonella typhimurium delta cya delta crp oral vaccine strains expressing a streptococcal colonization and virulence antigen." VACCINE, (1988 APR) 6 (2) 155-60. REF: 41 JOURNAL CODE: X60. ISSN: 0264-410X., XP002100818 ENGLAND: United Kingdom	1-4,6,7, 9,13-15, 17-19, 24-27, 29,30, 36-38, 40-42, 47-49, 51-53, 58-60, 62-64
X	see the whole document	68-71, 73-75, 80-86
Y	noting for example page 157, paragraph 3 – page 158, paragraph 1	12,23, 35,46, 57,67,79

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 April 1999	06/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CURTISS R 3D ET AL: "Selective delivery of antigens by recombinant bacteria." CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, (1989) 146 35-49. REF: 111 JOURNAL CODE: DWQ. ISSN: 0070-217X., XP002100819 GERMANY, WEST: Germany, Federal Republic of	1-4,6,7, 9,13-15, 17-20, 24-27, 29,30, 32, 36-38, 40-43, 47-49, 51-54, 58-60
X	see the whole document	62-64, 68-71, 73-76, 80-86
Y		12,23, 35,46, 57,67,79

X	WO 94 24291 A (UNIV WASHINGTON) 27 October 1994	1-4,6,7, 9,13-15, 17-19, 24-27, 29,30, 36-38, 40-42, 47-49, 51-53, 58-60, 62-64
X	see page 3, line 21 - page 5, line 2	68-71, 73-75, 80-86
Y		12,23, 35,46, 57,67,79

	-/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 09684 A (UNIV WASHINGTON) 11 June 1992	1-4,6,7, 9-11, 13-15, 17,21, 22, 24-27, 33,34, 36-38, 40,44, 45, 47-49, 51,55
X	see page 6, line 25 - page 7, line 26	56, 58-60, 62,65, 66, 68-71, 73,77, 78,80-86
Y	see page 28, line 33 - page 30, line 29	12,23, 35,46, 57,67,79
Y	WO 94 27634 A (TVW TELETHON INST CHILD HEALTH) 8 December 1994 see page 2, line 23 - line 29	12,23, 35,46, 57,67,79
X A	NICKERSON ET AL: "AN RPOS MUTANT OF S. TYPHIMURIUM IS DEFECTIVE IN THE INITIAL STAGES OF COLONIZATION AND IS IMMUNOGENIC IN MICE" ABSTRACTS OF THE 96TH GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, 1996, page 179 XP002100820 cited in the application see abstract B-141	87-90
X A	NICKERSON ET AL: "ROLE OF SIGMA FACTOR RPOS IN INITIAL STAGES OF SALMONELLA TYPHIMURIUM INFECTION" INFECTION AND IMMUNITY, vol. 65, no. 5, May 1997, pages 1814-1823, XP002100821 cited in the application see page 1814 see abstract	87-90

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

1

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   1-23, 81-83
    because they relate to subject matter not required to be searched by this Authority, namely:
    Remark: Although claims 1-23, 81-83
            are directed to a method of treatment of the human/animal
            body, the search has been carried out and based on the alleged
            effects of the compound/composition.

2. [ ] Claims Nos.:
    because they relate to parts of the International Application that do not comply with the prescribed requirements to such
    an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
    because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
    searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
    of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report
    covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
    restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                                   [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9424291	A	27-10-1994	AU	6705594 A	08-11-1994
WO 9209684	A	11-06-1992	AT	177787 T	15-04-1999
			AU	9094191 A	25-06-1992
			CA	2096529 A	22-05-1992
			DE	69131014 D	22-04-1999
			EP	0558631 A	08-09-1993
			IL	100121 A	15-06-1998
			US	5656488 A	12-08-1997
			CN	1072454 A	26-05-1993
WO 9427634	A	08-12-1994	AU	674584 B	02-01-1997
			AU	6838594 A	20-12-1994
			CA	2164326 A	08-12-1994
			EP	0705108 A	10-04-1996
			JP	9509135 T	16-09-1997
			NZ	266739 A	27-07-1997